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(54) Title: METHOD FOR DOWN-REGULATING OSTEOPROTEGERIN LIGAND ACTIVITY

(57) Abstract

The invention provides a novel method for down–regulating the biological activity of osteoprotegerin ligand (OPGL, TRANCE) thereby rendering possible the treatment/amelioration of diseases characterized by excessive loss of bone mass, e.g. osteoporosis. Down–regulation is effected by inducing an immune response against OPGL in an individual in need thereof. Immune responses can be raised by classical immunization with immunogenic variants of OPGL or by nucleic acid immunization where the nucleic acids encode the OPGL variant. The invention also pertains to compositions, polypeptides and nucleic acids useful in the invention, as well as to vectors and transformed host cells useful in the preparation thereof.

METHOD FOR DOWN-REGULATING OSTEOPROTEGERIN LIGAND ACTIVITY

FIELD OF THE INVENTION

The present invention relates to improvements in therapy and
prevention of osteoporosis and other diseases characterized by
5  continued loss of bone tissue. More specifically, the present
invention provides a method for down-regulating osteoprotege-
rin ligand (OPGL) by enabling the production of antibodies
against OPGL in subjects suffering from or in danger of suf-
fering from osteoporosis. The invention also provides for
10  methods of producing modified OPGL useful in this method as
well as for the modified OPGL as such. Also encompassed by the
present invention are nucleic acid fragments encoding modified
OPGL as well as vectors incorporating these nucleic acid
fragments and host cells and cell lines transformed therewith.
15  The invention also provides for a method for the identifica-
tion of OPGL analogues which are useful in the method of the
invention as well as for compositions comprising modified OPGL
or comprising nucleic acids encoding the OPGL analogues.

FIELD OF THE INVENTION

20  Osteoporosis is a major and growing health problem worldwide.
It affects an estimated 75 million people in the United States
of America, Europe and Japan combined. Thus, it is the most
common systemic bone disorder in the industrialised part of
the world.

25  Osteoporosis affects one in four postmenopausal women and a
majority of the elderly, including a substantial number of
men. The cost of osteoporosis in the United States of America
with 15 million affected people was estimated to be 3.8 bil-
lion USD annually in 1984. This translates by extrapolation to
30  a worldwide cost of something in the order of at least 20
billion USD.

2

Osteoporosis is a systemic skeletal disease characterised by
low bone mass and micro-architectural deterioration of bone
tissue, with a consequent increase in bone fragility and
susceptibility to fractures. Although all bones are affected,
5   fractures of the spine, wrist and hip are typical and the most
common. The risk of developing osteoporosis increases with age
and is higher in women than in men. Its etiology appears to
reside in the mechanisms underlying an accentuation of the
normal loss of bone mass, which follows the menopause in women
10  and occurs in all individuals with advancing age.

Peak bone mass is achieved at about 35 years of age. After
reaching its peak, bone mass declines throughout life due to
an imbalance in remodelling. Bones lose both mineral and
organic matrix but retain their basic organisation.

15  Bone consists of a mineralised extracellular matrix composed
of a variety of proteins and proteoglycans; the principal
component being type I collagen. The mineral encrusting the
extracellular matrix is hydroxyapatite $(Ca_3(PO_4)_2 \cdot Ca(OH)_2)$. Bone
is continuously modelled during growth and development and
20  remodelled throughout life in response to physical and chemi-
cal signals.

The growth, development and maintenance of bone are highly
regulated processes, which at the cellular level involves the
co-ordinate regulation of bone-forming cells (osteoblasts) and
25  bone-resorbing cells (osteoclasts). The level of bone mass
reflects the balance of bone formation and resorption.

Osteoblasts arise from mesenchymal stem cells and produce bone
matrix during development, after bone injury, and during the
normal bone remodelling that occurs throughout life. Osteo-
30  clasts differentiate from hematopoietic precursors of the
monocyte-macrophage lineage and resorb bone matrix.

3

An imbalance of osteoblast and osteoclast functions can result in the skeletal abnormalities characterised by increased bone mass (osteopetrosis) or by decreased bone mass (osteoporosis).

Studies of osteopetrosis in mutant mice have shown that ge-
5 netic defects in osteoclast development, maturation, and/or
activation lead to decreased bone resorption and uniformly
result in severe osteopetrosis (Marks, 1989). Nevertheless,
relatively little has so far been known about the soluble
factors that act physiologically to regulate osteoclast deve-
10 lopment.

Recently, however, two proteins that take part in this regula-
tion have been described and characterized (Simonet et al.,
1997; Lacey et al., 1998). These two proteins are
osteoprotegerin and osteoprotegerin ligand.

15 Osteoprotegerin is a novel secreted member of the tumour
necrosis factor receptor family. In vitro, osteoprotegerin
blocks osteoclastogenesis in a dose dependent manner. Trans-
genic mice expressing osteoprotegerin exhibit a generalized
increase in bone density (osteopetrosis) associated with a
20 decrease in osteoclasts. Administration of recombinant
osteoprotegerin produces similar effects in normal mice and
protects against ovariectomy-associated bone loss in rats
(Simonet et al., 1997). In addition, osteoprotegerin-deficient
mice (knock out mice) while normal at birth develop early
25 onset osteoporosis and arterial calcification (Bucay et al.,
1998). These observations strongly point to the possibility
that osteoprotegerin blocks the differentiation of osteo-
clasts, the principal if not sole bone-resorbing cell type,
suggesting that it can act as a humoral regulator of bone
30 resorption. Osteoprotegerin is the subject matter of WO
97/23614.

It was hypothesized that osteoprotegerin may exert its effect
by binding to and neutralising a factor that stimulates osteo-
clast development, thus inhibiting osteoclast maturation
(Simonet *et al.*, 1997).

5 Osteoprotegerin ligand (OPGL) is a novel member of the tumour
necrosis factor family of cytokines that exists in both a
membrane-bound and a soluble form. OPGL binds to osteoprote-
gerin with a binding affinity of 4 nM. *In vitro*, OPGL acti-
vates mature osteoclasts and modulates osteoclast formation
10 from bone marrow precursors in the presence of CSF-1. It has
also been demonstrated that OPGL binds to the surface of
osteoclast progenitors in CSF-1-treated bone marrow. The
receptor for OPGL on these hematopoeitic progenitor cells is,
however, unknown. Recombinant soluble OPGL is a potent inducer
15 of bone resorption *in vivo* (Lacey *et al.*, 1998).

Description of OPGL

OPGL is synthesised as a type II transmembrane protein consis-
ting of 317 amino acid residues (human, cf. SEQ ID NO: 2) or
316 amino acid residues (murine, cf. SEQ ID NOs: 4 and 6).
20 Alignment of the two amino acid sequences show that identical
amino acid residues are found at 87% of the homologous posi-
tions.

The OPGL amino acid sequence contains a short cytoplasmic
domain in the N-terminus followed by the putative transmem-
25 brane region between amino acid residues 49 and 69. Based on
its homology to tumour necrosis factor alpha, the extracellu-
lar part of OPGL has been suggested to be comprised by two
domains: a stalk region extending from amino acid residue 70
to 157, and the active ligand moiety extending from amino acid
30 residue 158 to the C-terminus.

The most closely related protein to OPGL appears to be the
apoptosis inducing cytokine TRAIL with less that 25% identical
amino acid residues. OPGL has also very recently been cloned
in other contexts and was called TRANCE (Wong et al., 1997, J.
5  Biol. Chem. **272**: 25190-25194) and RANKL, respectively (Ander-
son et al., 1997, Nature **390**: 175-179. The protein is also
known as osteoclast differentiation factor (ODF).

Several N-terminal deletion variants of murine OPGL have been
expressed in E. coli and purified. These variants consisted of
10  amino acid residues 75-316, 128-316, 137-316, and 158-316,
respectively. The three shortest variants had similar β-sheet
structure based on circular dichroism studies, and all were
able to bind to osteoprotegerin. More important, though, is
that the three variants were active in in vitro assays (Lacey
15  et al., 1998).

The shortest variant was studied further. Like tumour necrosis
factor alpha, this variant OPGL exists as a trimer in solution
and forms 3:3 complexes when incubated with osteoprotegerin.
The binding affinity was found to be 4 nM. This variant in-
20  duces significant increases in blood ionized calcium
(hypercalcemia) in mice in vivo. Co-administration of
osteoprotegerin significantly reduced this hypercalcemic
effect of OPGL.

The longest variant (amino acid residues 75-316) of OPGL did
25  not bind to osteoprotegerin and it did not have any biological
activity.

At the time of construction of the N-terminal deletion vari-
ants the natural cleavage site in OPGL was not known. Expres-
sion of full-length OPGL in human 293 fibroblasts resulted in
30  soluble OPGL beginning at amino residue 139 in the murine
protein or at the homologous amino acid residue 140 in the

human protein. These expression studies also showed that
soluble OPGL resulting from expression in human cells is
glycosylated. This is not surprising as both murine and human
OPGL contain three potential N-glycosylation sites in the C-
5  terminal ligand domain.

The concentrations of osteoprotegerin in blood and tissues are
not known but the protein has significant biological activity
at a concentration of 1 ng/ml.

Biological activity of OPGL

10  OPGL is a potent osteoclast differentiation factor when com-
bined with CSF-1. Neither of these components alone are cap-
able of inducing osteoclast differentiation from progenitor
cells.

OPGL is a potent activator of mature osteoclast. On its own,
15  OPGL activates mature osteoclasts to resorb bone. OPGL has not
been observed to act as an osteoclast growth factor or osteo-
clast survival factor in these experiments.

The action of OPGL does not seem to be species restricted as
murine OPGL also induced osteoclast formation in cultures of
20  human peripheral blood mononuclear cells.

OBJECT OF THE INVENTION

The object of the present invention is to provide novel thera-
pies against conditions characterized by excess bone resorp-
tion, such as osteoporosis. A further object is to develop an
25  autovaccine against OPGL, in order to obtain a novel treatment
for osteoporosis and for other pathological disorders invol-
ving excess bone resorption.

## SUMMARY OF THE INVENTION

We find that the above-referenced data suggests a
pathophysiological role of OPGL. The *in vivo* evidence is par-
tially circumstantial or indirect but is in our opinion
5  convincing especially in combination with the direct evidence.

Observing that injection into mice of the recombinant C-termi-
nal domain of OPGL results in severe hypercalcemia in our
opinion points directly to a pathophysiological role.

Indirect evidence comes from the osteoprotegerin-deficient
10  mice (knock out mice) that even though normal at birth develop
early onset osteoporosis. This shows that removing a protein
that binds OPGL and neutralises its effects leads to osteopo-
rosis. We conclude that the most likely reason for this is an
increased osteoclast maturation and activation caused by OPGL.

15  Two other pieces of indirect evidence are that both mice
transgenic for osteoprotegerin and mice injected with recombi-
nant osteoprotegerin develop osteopetrosis. This shows that
unnatural high levels of a protein that binds OPGL and neu-
tralises its effects leads to osteopetrosis. Here, we conclude
20  that this has its reasons in a decreased osteoclast maturation
and activation caused by neutralisation of OPGL.

We therefore suggest a model in which OPGL and osteoprotegerin
act as positive and negative regulators of osteoclast develop-
ment, respectively. In other words OPGL promotes bone resorp-
25  tion while osteoprotegerin inhibits bone resorption.

Thus, in relation to osteoporosis OPGL could be thought of as
a "pathogenic agent" which promotes the bone resorption that
in the end leads to osteoporosis. Likewise osteoprotegerin can

be visualised as a "therapeutic agent" which counteracts the
"pathogenic agent" through neutralisation of its effects.

We hence propose to down-regulate osteoclast differentia-
tion/maturation/formation and osteoclast activation through *in*
5  *vivo* production of antibodies capable of neutralizing OPGL,
thereby providing a safe and efficient means for treating/a-
meliorating and/or preventing osteoporosis and other diseases
characterized by an excess rate of bone resorption compared to
the rate of bone formation.

10 Thus, in its broadest and most general scope, the present
invention relates to a method for *in vivo* down-regulation of
osteoprotegerin ligand (OPGL) activity in an animal, including
a human being, the method comprising effecting presentation to
the animal's immune system of an immunologically effective
15 amount of
   -    at least one OPGL polypeptide or subsequence thereof
        which has been formulated so that immunization of the
        animal with the OPGL polypeptide or subsequence thereof
        induces production of antibodies against the OPGL
20      polypeptide, and/or
   -    at least one OPGL analogue wherein is introduced a
        modification in the OPGL polypeptide which has as a re-
        sult that immunization of the animal with the analogue
        induces production of antibodies against the OPGL
25      polypeptide.

The most attractive aspect of this approach is that e.g.
osteoporosis can be controlled by periodic but not very fre-
quent immunizations, in contrast to a therapeutic approach
which involves frequent (e.g. daily) administration of
30 osteoprotegerin or molecules having a binding affinity to OPGL
analogous therewith. It is expected that 1-4 annual injections
with an immunogenic composition will be sufficient to obtain

the desired effect, whereas administration of osteoprotegerin
or other inhibitors of OPGL activity would require daily
administrations.

The invention also relates to OPGL analogues as well as to
5  nucleic acid fragments encoding a subset of these. Also    —
immunogenic compositions comprising the analogues or the
nucleic acid fragments are part of the invention.

The invention also relates to a method of identifying ana-
logues of OPGL as well as a method for preparing composition
10 comprising the OPGL analogues.

Finally, the invention relates to a method treating osteoporo-
sis and other diseases characterized in excess bone resorp-
tion, wherein is administered a non-OPGL molecule (typically
an antibody) which blocks the interaction between OPGL and its
15 receptor on osteoclast cells.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following a number of terms used in the present
specification and claims will be defined and explained in
20 detail in order to clarify the metes and bounds of the inven-
tion.

The terms "T-lymphocyte" and "T-cell" will be used
interchangeably for lymphocytes of thymic origin which are
responsible for various cell mediated immune responses as well
25 as for helper activity in the humoral immune response. Like-
wise, the terms "B-lymphocyte" and "B-cell" will be used
interchangeably for antibody-producing lymphocytes.

An "OPGL polypeptide" is herein intended to denote polypep-
tides having the amino acid sequence of the above-discussed
OPGL proteins derived from humans and mice (or truncates
thereof sharing a substantial amount of B-cell epitopes with
5   intact OPGL), but also polypeptides having the amino acid
sequence identical to analogues of these two proteins isolated
from other species are embraced by the term. Also unglycosyla-
ted forms of OPGL which are prepared in prokaryotic system are
included within the boundaries of the term as are forms having
10  varying glycosylation patterns due to the use of e.g. yeasts
or other non-mammalian eukaryotic expression systems. It
should, however, be noted that when using the term "an OPGL
polypeptide" it is intended that the polypeptide in question
is normally non-immunogenic when presented to the animal to be
15  treated. In other words, the OPGL polypeptide is a self-pro-
tein or is an analogue of such a self-protein which will not
normally give rise to an immune response against OPGL of the
animal in question.

An "OPGL analogue" is an OPGL polypeptide which has been
20  subjected to changes in its primary structure. Such a change
can e.g. be in the form of fusion of an OPGL polypeptide to a
suitable fusion partner (i.e. a change in primary structure
exclusively involving C- and/or N-terminal additions of amino
acid residues) and/or it can be in the form of insertions
25  and/or deletions and/or substitutions in the OPGL polypep-
tide's amino acid sequence. Also encompassed by the term are
derivatized OPGL molecules, cf. the discussion below of modi-
fications of OPGL.

It should be noted that the use as a vaccine in a human of a
30  xeno-analogue (e.g. a canine or porcine analogue) of human
OPGL can be imagined to produce the desired immunity against
OPGL. Such use of an xeno-analogue for immunization is also
considered part of the invention.

The term "polypeptide" is in the present context intended to mean both short peptides of from 2 to 10 amino acid residues, oligopeptides of from 11 to 100 amino acid residues, and polypeptides of more than 100 amino acid residues. Further-
5  more, the term is also intended to include proteins, *i.e.* functional biomolecules comprising at least one polypeptide; when comprising at least two polypeptides, these may form complexes, be covalently linked, or may be non-covalently linked. The polypeptide(s) in a protein can be glycosylated
10  and/or lipidated and/or comprise prosthetic groups.

The term "subsequence" means any consecutive stretch of at least 3 amino acids or, when relevant, of at least 3 nucleo-tides, derived directly from a naturally occurring OPGL amino acid sequence or nucleic acid sequence, respectively.

15  The term "animal" is in the present context in general in-tended to denote an animal species (preferably mammalian), such as *Homo sapiens*, *Canis domesticus*, etc. and not just one single animal. However, the term also denotes a population of such an animal species, since it is important that the indi-
20  viduals immunized according to the method of the invention all harbour substantially the same OPGL allowing for immunization of the animals with the same immunogen(s). If, for instance, genetic variants of OPGL exists in different human population it may be necessary to use different immunogens in these
25  different populations in order to be able to break the autotolerance towards OPGL in each population. It will be clear to the skilled person that an animal in the present context is a living being which has an immune system. It is preferred that the animal is a vertebrate, such as a mammal.

30  By the term "*in vivo* down-regulation of OPGL activity" is herein meant reduction in the living organism of the number of interactions between OPGL and its (unknown) receptor (or

between OPGL and other possible biologically important binding partners for this molecule). The down-regulation can be obtained by means of several mechanisms: Of these, simple interference with the active site in OPGL by antibody binding is

5  the most simple. However, it is also within the scope of the present invention that the antibody binding results in removal of OPGL by scavenger cells (such as macrophages and other phagocytic cells).

The expression "effecting presentation ... to the immune
10  system" is intended to denote that the animal's immune system is subjected to an immunogenic challenge in a controlled manner. As will appear from the disclosure below, such challenge of the immune system can be effected in a number of ways of which the most important are vaccination with polypeptide
15  containing "pharmaccines" (i.e. a vaccine which is administered to treat or ameliorate ongoing disease) or nucleic acid "pharmaccine" vaccination. The important result to achieve is that immune competent cells in the animal are confronted with the antigen in an immunologically effective manner, whereas
20  the precise mode of achieving this result is of less importance to the inventive idea underlying the present invention.

The term "immunogenically effective amount" has its usual meaning in the art, i.e. an amount of an immunogen which is capable of inducing an immune response which significantly
25  engages pathogenic agents which share immunological features with the immunogen.

When using the expression that the OPGL has been "modified" is herein meant a chemical modification of the polypeptide which constitutes the backbone of OPGL. Such a modification can e.g.
30  be derivatization (e.g. alkylation) of certain amino acid residues in the OPGL sequence, but as will be appreciated from the disclosure below, the preferred modifications comprise

changes of the primary structure of the OPGL amino acid se-
quence.

When discussing "autotolerance towards OPGL" it is understood
that since OPGL is a self-protein in the population to be
5  vaccinated, normal individuals in the population do not mount
an immune response against OPGL; it cannot be excluded,
though, that occasional individuals in an animal population
might be able to produce antibodies against native OPGL, e.g.
as part of a autoimmune disorder. At any rate, an animal will
10 normally only be autotolerant towards its own OPGL, but it
cannot be excluded that OPGL analogues derived from other
animal species or from a population having a different OPGL
phenotype would also be tolerated by said animal.

A "foreign T-cell epitope" (or: "foreign T-lymphocyte
15 epitope") is a peptide which is able to bind to an MHC
molecule and which stimulates T-cells in an animal species.
Preferred foreign T-cell epitopes in the invention are
"promiscuous" epitopes, i.e. epitopes which bind to a
substantial fraction of a particular class of MHC molecules in
20 an animal species or population. Only a very limited number of
such promiscuous T-cell epitopes are known, and they will be
discussed in detail below. It should be noted that in order
for the immunogens which are used according to the present
invention to be effective in as large a fraction of an animal
25 population as possible, it may be necessary to 1) insert
several foreign T-cell epitopes in the same OPGL analogue or
2) prepare several OPGL analogues wherein each analogue has a
different promiscuous epitope inserted. It should be noted
also that the concept of foreign T-cell epitopes also
30 encompasses use of cryptic T-cell epitopes, i.e. epitopes
which are derived from a self-protein and which only exerts
immunogenic behaviour when existing in isolated form without
being part of the self-protein in question.

A "foreign T helper lymphocyte epitope" (a foreign $T_H$ epitope)
is a foreign T cell epitope which binds an MHC Class Class II
molecule and can be presented on the surface of an antigen
presenting cell (APC) bound to the MHC Class II molecule.

5   A "functional part" of a (bio)molecule is in the present    —
context intended to mean the part of the molecule which is
responsible for at least one of the biochemical or physiologi-
cal effects exerted by the molecule. It is well-known in the
art that many enzymes and other effector molecules have an
10  active site which is responsible for the effects exerted by
the molecule in question. Other parts of the molecule may
serve a stabilizing or solubility enhancing purpose and can
therefore be left out if these purposes are not of relevance
in the context of a certain embodiment of the present inven-
15  tion. For instance it is possible to use certain cytokines as
a modifying moiety in OPGL (cf. the detailed discussion be-
low), and in such a case, the issue of stability may be irrel-
evant since the coupling to OPGL provides the stability neces-
sary.

20  The term "adjuvant" has its usual meaning in the art of vac-
cine technology, i.e. a substance or a composition of matter
which is 1) not in itself capable of mounting a specific
immune response against the immunogen of the vaccine, but
which is 2) nevertheless capable of enhancing the immune
25  response against the immunogen. Or, in other words, vaccina-
tion with the adjuvant alone does not provide an immune re-
sponse against the immunogen, vaccination with the immunogen
may or may not give rise to an immune response against the
immunogen, but the combined vaccination with immunogen and
30  adjuvant induces an immune response against the immunogen
which is stronger than that induced by the immunogen alone.

"Targeting" of a molecule is in the present context intended
to denote the situation where a molecule upon introduction in
the animal will appear preferentially in certain tissue(s) or
will be preferentially associated with certain cells or cell
5   types. The effect can be accomplished in a number of ways
including formulation of the molecule in composition facili--
tating targeting or by introduction in the molecule of groups
which facilitates targeting. These issues will be discussed in
detail below.

10   "Stimulation of the immune system" means that a substance or
composition of matter exhibits a general, non-specific
immunostimulatory effect. A number of adjuvants and putative
adjuvants (such as certain cytokines) share the ability to
stimulate the immune system. The result of using an
15   immunostimulating agent is an increased "alertness" of the
immune system meaning that simultaneous or subsequent immuni-
zation with an immunogen induces a significantly more effec-
tive immune response compared to isolated use of the immunogen

Preferred embodiments of OPGL activity down-regulation

20   It is preferred that the OPGL polypeptide used as an immunogen
in the method of the invention is a modified molecule wherein
at least one change is present in the OPGL amino acid sequen-
ce, since the chances of obtaining the all-important breaking
of autotolerance towards OPGL is greatly facilitated that way.
25   It should be noted that this does not exclude the possibility
of using such a modified OPGL in formulations which further
facilitate the breaking of autotolerance against OPGL, e.g.
formulations containing adjuvants.

It has been shown (in Dalum I et al., 1996, J. Immunol. 157:
30   4796-4804) that potentially self-reactive B-lymphocytes recog-
nizing self-proteins are physiologically present in normal

individuals. However, in order for these B-lymphocytes to be
induced to actually produce antibodies reactive with the rele-
vant self-proteins, assistance is needed from cytokine produ-
cing T-helper lymphocytes ($T_H$-cells or $T_H$-lymphocytes). Nor-
5   mally this help is not provided because T-lymphocytes in
general do not recognize T-cell epitopes derived from self-
proteins when presented by antigen presenting cells (APCs).
However, by providing an element of "foreignness" in a self-
protein (i.e. by introducing an immunologically significant
10  modification), T-cells recognizing the foreign element are
activated upon recognizing the foreign epitope on an APC (such
as, initially, a mononuclear cell). Polyclonal B-lymphocytes
(which are also APCs) capable of recognising self-epitopes on
the modified self-protein also internalise the antigen and
15  subsequently presents the foreign T-cell epitope(s) thereof,
and the activated T-lymphocytes subsequently provide cytokine
help to these self-reactive polyclonal B-lymphocytes. Since
the antibodies produced by these polyclonal B-lymphocytes are
reactive with different epitopes on the modified polypeptide,
20  including those which are also present in the native polypep-
tide, an antibody cross-reactive with the non-modified self-
protein is induced. In conclusion, the T-lymphocytes can be
led to act as if the population of polyclonal B-lymphocytes
have recognised an entirely foreign antigen, whereas in fact
25  only the inserted epitope(s) is/are foreign to the host. In
this way, antibodies capable of *cross-reacting* with non-modi-
fied self-antigens are induced.

Several ways of modifying a peptide self-antigen in order to
obtain breaking of autotolerance are known in the art. Hence,
30  according to the invention, the modification can include that

-    at least one foreign T-cell epitope is introduced, and/or

- at least one first moiety is introduced which effects targeting of the modified molecule to an antigen presenting cell (APC), and/or
- at least one second moiety is introduced which stimulates
5   the immune system, and/or
- at least one third moiety is introduced which optimizes⁻ presentation of the modified OPGL polypeptide to the immune system.

However, all these modifications should be carried out while
10  maintaining a substantial fraction of the original B-lymphocyte epitopes in OPGL, since the B-lymphocyte recognition of the native molecule is thereby enhanced.

In one preferred embodiment, side groups (in the form of foreign T-cell epitopes or the above-mentioned first, second
15  and third moieties) are covalently or non-covalently introduced. This is to mean that stretches of amino acid residues derived from OPGL are derivatized without altering the primary amino acid sequence, or at least without introducing changes in the peptide bonds between the individual amino acids in the
20  chain.

An alternative, and preferred, embodiment utilises amino acid substitution and/or deletion and/or insertion and/or addition (which may be effected by recombinant means or by means of peptide synthesis; modifications which involves longer
25  stretches of amino acids can give rise to fusion polypeptides). One especially preferred version of this embodiment is the technique described in WO 95/05849, which discloses a method for down-regulating self-proteins by immunising with analogues of the self-proteins wherein a number of amino acid
30  sequence(s) has been substituted with a corresponding number of amino acid sequence(s) which each comprise a foreign immunodominant T-cell epitope, while at the same time maintaining the overall tertiary structure of the self-protein in

the analogue. For the purposes of the present invention, it is
however sufficient if the modification (be it an insertion,
addition, deletion or substitution) gives rise to a foreign T-
cell epitope and at the same time preserves a substantial
5   number of the B-cell epitopes in OPGL. However, in order to
obtain maximum efficacy of the immune response induced, it is
preferred that the overall tertiary structure of OPGL is
maintained in the modified molecule.

The following formula describes the OPGL constructs generally
10  covered by the invention:

$$(MOD_1)_{s1}(OPGL_{e1})_{n1}(MOD_2)_{s2}(OPGL_{e2})_{n2}\ldots(MOD_x)_{sx}(OPGL_{ex})_{nx} \qquad (I)$$

-where $OPGL_{e1}$-$OPGL_{ex}$ are x B-cell epitope containing subsequen-
ces of OPGL which independently are identical or non-identical
and which may contain or not contain foreign side groups, x is
15  an integer $\geq 3$, n1-nx are x integers $\geq 0$ (at least one is $\geq$
1), $MOD_1$-$MOD_x$ are x modifications introduced between the pre-
served B-cell epitopes, and $s_1$-$s_x$ are x integers $\geq 0$ (at least
one is $\geq 1$ if no side groups are introduced in the $OPGL_e$ se-
quences). Thus, given the general functional restraints on the
20  immunogenicity of the constructs, the invention allows for all
kinds of permutations of the original OPGL sequence, and all
kinds of modifications therein. Thus, included in the inven-
tion are modified OPGL obtained by omission of parts of the
OPGL sequence which e.g. exhibit adverse effects *in vivo* or
25  omission of parts which are normally intracellular and thus
could give rise to undesired immunological reactions.

Maintenance of a substantial fraction of B-cell epitopes or
even the overall tertiary structure of a protein which is
30  subjected to modification as described herein can be achieved
in several ways. One is simply to prepare a polyclonal anti-
serum directed against OPGL (e.g. an antiserum prepared in a

rabbit) and thereafter use this antiserum as a test reagent (e.g. in a competitive ELISA) against the modified proteins which are produced. Modified versions (analogues) which react to the same extent with the antiserum as does OPGL must be

5 regarded as having the same overall tertiary structure as OPGL whereas analogues exhibiting a limited (but still significant and specific) reactivity with such an antiserum are regarded as having maintained a substantial fraction of the original B-cell epitopes.

10 Alternatively, a selection of monoclonal antibodies reactive with distinct epitopes on OPGL can be prepared and used as a test panel. This approach has the advantage of allowing 1) an epitope mapping of OPGL and 2) a mapping of the epitopes which are maintained in the analogues prepared.

15 Of course, a third approach would be to resolve the 3-dimensional structure of OPGL or of a biologically active truncate thereof (cf. above) and compare this to the resolved three-dimensional structure of the analogues prepared. Three-dimensional structure can be resolved by the aid of X-ray diffrac-

20 tion studies and NMR-spectroscopy. Further information relating to the tertiary structure can to some extent be obtained from circular dichroism studies which have the advantage of merely requiring the polypeptide in pure form (whereas X-ray diffraction requires the provision of crystallized polypeptide

25 and NMR requires the provision of isotopic variants of the polypeptide) in order to provide useful information about the tertiary structure of a given molecule. However, ultimately X-ray diffraction and/or NMR are necessary to obtain conclusive data since circular dichroism can only provide indirect evi-

30 dence of correct 3-dimensional structure via information of secondary structure elements.

One preferred embodiment of the invention utilises multiple
presentations of B-lymphocyte epitopes of OPGL (*i.e.* formula I
wherein at least one B-cell epitope is present in two posi-
tions). This effect can be achieved in various ways, e.g. by
5   simply preparing fusion polypeptides comprising the structure
(OPGL)$_m$, where m is an integer ≥ 2 and then introduce the modi-
fications discussed herein in at least one of the OPGL sequen-
ces. It is preferred that the modifications introduced in-
cludes at least one duplication of a B-lymphocyte epitope
10  and/or the introduction of a hapten.

As mentioned above, the introduction of a foreign T-cell
epitope can be accomplished by introduction of at least one
amino acid insertion, addition, deletion, or substitution. Of
course, the normal situation will be the introduction of more
15  than one change in the amino acid sequence (e.g. insertion of
or substition by a complete T-cell epitope) but the important
goal to reach is that the OPGL analogue, when processed by an
antigen presenting cell (APC), will give rise to such a for-
eign immunodominant T-cell epitope being presented in context
20  of an MCH Class II molecule on the surface of the APC. Thus,
if the OPGL amino acid sequence in appropriate positions
comprises a number of amino acid residues which can also be
found in a foreign T$_H$ epitope then the introduction of a for-
eign T$_H$ epitope can be accomplished by providing the remaining
25  amino acids of the foreign epitope by means of amino acid
insertion, addition, deletion and substitution. In other
words, it is not necessary to introduce a complete T$_H$ epitope
by insertion or substitution in order to fulfill the purpose
of the present invention.

30  It is preferred that the number of amino acid insertions,
deletions, substitutions or additions is at least 2, such as
3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,

20, and 25 insertions, substitutions, additions or deletions.
It is furthermore preferred that the number of amino acid
insertions, substitutions, additions or deletions is not in
excess of 150, such as at most 100, at most 90, at most 80,
5  and at most 70. It is especially preferred that the number of
substitutions, insertions, deletions, or additions does not ⁻
exceed 60, and in particular the number should not exceed 50
or even 40. Most preferred is a number of not more than 30.
With respect to amino acid additions, it should be noted that
10 these, when the resulting construct is in the form of a fusion
polypeptide, is often considerably higher than 150.


Preferred embodiments of the invention includes modification
by introducing at least one foreign immunodominant T-cell
epitope. It will be understood that the question of immune
15 dominance of a T-cell epitope depends on the animal species in
question. As used herein, the term "immunodominance" simply
refers to epitopes which in the vaccinated individual/po-
pulation gives rise to a significant immune response, but it
is a well-known fact that a T-cell epitope which is
20 immunodominant in one individual/population is not necessarily
immunodominant in another individual of the same species, even
though it may be capable of binding MHC-II molecules in the
latter individual. Hence, for the purposes of the present
invention, an immune dominant T-cell epitope is a T-cell
25 epitope which will be effective in providing T-cell help when
present in an antigen. Typically, immune dominant T-cell
epitopes has as an inherent feature that they will substan-
tially always be presented bound to an MHC Class II molecule,
irrespective of the polypeptide wherein they appear.


30 Another important point is the issue of MHC restriction of T-
cell epitopes. In general, naturally occurring T-cell epitopes
are MHC restricted, *i.e.* a certain peptides constituting a T-
cell epitope will only bind effectively to a subset of MHC

Class II molecules. This in turn has the effect that in most cases the use of one specific T-cell epitope will result in a vaccine component which is only effective in a fraction of the population, and depending on the size of that fraction, it can

5    be necessary to include more T-cell epitopes in the same molecule, or alternatively prepare a multi-component vaccine wherein the components are OPGL variants which are distinguished from each other by the nature of the T-cell epitope introduced.

10   If the MHC restriction of the T-cells used is completely unknown (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula

15

$$f_{population} = 1 - \prod_{i=1}^{n} (1 - p_i) \qquad (II)$$

-where $p_i$ is the frequency in the population of responders to the $i^{th}$ foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in

20   the vaccine composition. Thus, a vaccine composition containing 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

1 - 0.2 × 0.3 × 0.4 = 0.976

-i.e. 97.6 percent of the population will statistically mount

25   an MHC-II mediated response to the vaccine.

The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and

DR7, then the use of this peptide together with another pep-
tide which binds the remaining MHC-II molecules encoded by
HLA-DR alleles will accomplish 100% coverage in the population
in question. Likewise, if the second peptide only binds DR3
5   and DR5, the addition of this peptide will not increase the
coverage at all. If one bases the calculation of population —
response purely on MHC restriction of T-cell epitopes in the
vaccine, the fraction of the population covered by a specific
vaccine composition can be determined by means of the follow-
10  ing formula:

$$f_{population} = 1 - \prod_{j=1}^{3} (1 - \varphi_j)^2 \qquad (III)$$

-wherein $\varphi_j$ is the sum of frequencies in the population of
allelic haplotypes encoding MHC molecules which bind any one
of the T-cell epitopes in the vaccine and which belong to the
15  $j^{th}$ of the 3 known HLA loci (DP, DR and DQ); in practice, it is
first determined which MHC molecules will recognize each T-
cell epitope in the vaccine and thereafter these are listed by
type (DP, DR and DQ) - then, the individual frequencies of the
different listed allelic haplotypes are summed for each type,
20  thereby yielding $\varphi_1$, $\varphi_2$, and $\varphi_3$.

It may occur that the value $p_i$ in formula II exceeds the corre-
sponding theoretical value $\pi_i$:

$$\pi_i = 1 - \prod_{j=1}^{3} (1 - v_j)^2 \qquad (IV)$$

-wherein $v_j$ is the sum of frequencies in the population of
25  allelic haplotype encoding MHC molecules which bind the $i^{th}$ T-
cell epitope in the vaccine and which belong to the $j^{th}$ of the
3 known HLA loci (DP, DR and DQ). This means that in $1-\pi_i$ of

24

the population is a frequency of responders of $f_{residual\_i} = (p_i - \pi_i)/(1-\pi_i)$. Therefore, formula III can be adjusted so as to yield formula V:

$$f_{population} = 1 - \prod_{j=1}^{3} (1-\varphi_j)^2 + \left(1 - \prod_{i=1}^{n} (1 - f_{residual\_i})\right) \qquad \underline{(V)}$$

5   -where the term $1-f_{residual-i}$ is set to zero if negative. It should be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the OPGL analogue, it is important to include all knowledge of
10  the epitopes which is available: 1) The frequency of responders in the population to each epitope, 2) MHC restriction data, and 3) frequency in the population of the relevant haplotypes.

There exist a number of naturally occurring "promiscuous" T-
15  cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these are preferably introduced in the vaccine thereby reducing the need for a very large number of different OPGL analogues in the same vaccine.

20  The promiscuous epitope can according to the invention be a naturally occurring human T-cell epitope such as epitopes from tetanus toxoid (e.g. the P2 and P30 epitopes), diphtheria toxoid, Influenza virus hemagluttinin (HA), and *P. falciparum* CS antigen.

25  Over the years a number of other promiscuous T-cell epitopes have been identified. Especially peptides capable of binding a large proportion of HLA-DR molecules encoded by the different HLA-DR alleles have been identified and these are all possible

T-cell epitopes to be introduced in the OPGL analogues used
according to the present invention. Cf. also the epitopes
discussed in the following references which are hereby all
incorporated by reference herein: WO 98/23635 (Frazer IH *et*
5  *al.*, assigned to The University of Queensland); Southwood S
*et. al*, 1998, J. Immunol. **160**: 3363-3373; Sinigaglia F *et al.*,
1988, Nature **336**: 778-780; Chicz RM *et al.*, 1993, J. Exp. Med
**178**: 27-47; Hammer J *et al.*, 1993, Cell **74**: 197-203; and Falk
K *et al.*, 1994, Immunogenetics **39**: 230-242. The latter refe-
10  rence also deals with HLA-DQ and -DP ligands. All epitopes
listed in these 5 references are relevant as candidate natural
epitopes to be used in the present invention, as are epitopes
which share common motifs with these.

Alternatively, the epitope can be any artificial T-cell epi-
15  tope which is capable of binding a large proportion of MHC
Class II molecues. In this context the pan DR epitope peptides
("PADRE") described in WO 95/07707 and in the corresponding
paper Alexander J *et al.*, 1994, Immunity **1**: 751-761 (both
disclosures are incorporated by reference herein) are inter-
20  esting candidates for epitopes to be used according to the
present invention. It should be noted that the most effective
PADRE peptides disclosed in these papers carry D-amino acids
in the C- and N-termini in order to improve stability when
administered. However, the present invention primarily aims at
25  incorporating the relevant epitopes as part of the modified
OPGL which should then subsequently be broken down enzymati-
cally inside the lysosomal compartment of APCs to allow subse-
quent presentation in the context of an MHC-II molecule and
therefore it is not expedient to incorporate D-amino acids in
30  the epitopes used in the present invention.

One especially preferred PADRE peptide is the one having the
amino acid sequence AKFVAAWTLKAAA or an immunologically effec-
tive subsequence thereof. This, and other epitopes having the

26

same lack of MHC restriction are preferred T-cell epitopes
which should be present in the OPGL analogues used in the
inventive method. Such super-promiscuous epitopes will allow
for the most simple embodiments of the invention wherein only
5 one single modified OPGL is presented to the vaccinated ani-
mal's immune system.

As mentioned above, the modification of OPGL can also include
the introduction of a first moiety which targets the modified
OPGL to an APC or a B-lymphocyte. For instance, the first
10 moiety can be a specific binding partner for a B-lymphocyte
specific surface antigen or for an APC specific surface anti-
gen. Many such specific surface antigens are known in the art.
For instance, the moiety can be a carbohydrate for which there
is a receptor on the B-lymphocyte or the APC (e.g. mannan or
15 mannose). Alternatively, the second moiety can be a hapten.
Also an antibody fragment which specifically recognizes a
surface molecule on APCs or lymphocytes can be used as a first
moiety (the surface molecule can e.g. be an FCγ receptor of
macrophages and monocytes, such as FCγRI or, alternatively any
20 other specific surface marker such as CD40 or CTLA-4). It
should be noted that all these exemplary targeting molecules
can be used as part of an adjuvant also, cf. below.

As an alternative or supplement to targeting the modified OPGL
polypeptide to a certain cell type in order to achieve an en-
25 hanced immune response, it is possible to increase the level
of responsiveness of the immune system by including the above-
mentioned second moiety which stimulates the immune system.
Typical examples of such second moieties are cytokines, and
heat-shock proteins or molecular chaperones, as well as effec-
30 tive parts thereof.

Suitable cytokines to be used according to the invention are
those which will normally also function as adjuvants in a vac-

cine composition, *i.e.* for instance interferon γ (IFN-γ),
interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-
4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin
13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage
5 colony stimulating factor (GM-CSF); alternatively, the func-
tional part of the cytokine molecule may suffice as the second
moiety. With respect to the use of such cytokines as adjuvant
substances, cf. the discussion below.

According to the invention, suitable heat-shock proteins or
10 molecular chaperones used as the second moiety can be HSP70,
HSP90, HSC70, GRP94 (also known as gp96, cf. Wearsch PA *et al.*
1998, Biochemistry **37**: 5709-19), and CRT (calreticulin).

Alternatively, the second moiety can be a toxin, such as
listeriolycin (LLO), lipid A and heat-labile enterotoxin.
15 Also, a number of mycobacterial derivatives such as MDP (mura-
myl dipeptide), CFA (complete Freund's adjuvant) and the
trehalose diesters TDM and TDE are interesting possibilities.

Also the possibility of introducing a third moiety which en-
hances the presentation of the modified OPGL to the immune
20 system is an important embodiment of the invention. The art
has shown several examples of this principle. For instance, it
is known that the palmitoyl lipidation anchor in the *Borrelia
burgdorferi* protein OspA can be utilised so as to provide
self-adjuvating polypeptides (cf. e.g. WO 96/40718) – it seems
25 that the lipidated proteins form up micelle-like structures
with a core consisting of the lipidation anchor parts of the
polypeptides and the remaining parts of the molecule protru-
ding therefrom, resulting in multiple presentations of the
antigenic determinants. Hence, the use of this and related
30 approaches using different lipidation anchors (e.g. a myristyl
group, a myristyl group, a farnesyl group, a geranyl-geranyl
group, a GPI-anchor, and an N-acyl diglyceride group) are

preferred embodiments of the invention, especially since the
provision of such a lipidation anchor in a recombinantly
produced protein is fairly straightforward and merely requires
use of e.g. a naturally occurring signal sequence as a fusion
5  partner for the modified OPGL polypeptide. Another possibility
is use of the C3d fragment of complement factor C3 or C3  —
itself (cf. Dempsey *et al.*, 1996, Science **271**, 348-350 and Lou
& Kohler, 1998, Nature Biotechnology **16**, 458-462).


An alternative embodiment of the invention which also results
10  in the preferred presentation of multiple (e.g. at least 2)
copies of the important epitopic regions of OPGL to the immune
system is the covalent coupling of OPGL, subsequence or vari-
ants thereof to certain molecules. For instance, polymers can
be used, e.g. carbohydrates such as dextran, cf. e.g. Lees A
15  *et al.*, 1994, Vaccine **12**: 1160-1166; Lees A *et al.*, 1990, J
Immunol. **145**: 3594-3600, but also mannose and mannan are
useful alternative. Integral membrane proteins from e.g. *E.
coli* and other bacteria are also useful conjugation partners.
The traditional carrier molecules such as keyhole limpet
20  hemocyanin (KLH), tetanus toxoid, diphtheria toxoid, and
bovine serum albumin (BSA) are also preferred and useful
conjugation partners.

Certain areas of native OPGL seems to be most suited for per-
forming modifications. Because of OPGL's structural relation-
25  ship with TNF-α and other members of the tumour necrosis
factor family, it is predicted that introductions of T-cell
epitopes or other modifications in areas defined by positions
170-192, 198-218, 221-246, 256-261, or 285-316, (the amino
acid numbering of SEQ ID NOs: 4, 6, and 12) will be most
30  likely to produce the desired results. These positions refer
to the murine OPGL - the corresponding positions in the human
molecule are 171-193, 199-219, 222-247, 257-262, and 286-317
(the amino acid numbering of SEQ ID NO: 2).

Considerations underlying these chosen areas are a) preserva-
tion of known and predicted B-cell epitopes, b) preservation
of tertiary structure etc. At any rate, as discussed above, it
is fairly easy to screen a set of modified OPGL molecules
5  which have all been subjected to introduction of a T-cell
epitope in different locations.

Since the most preferred embodiments of the present invention
involves down-regulation of human OPGL, it is consequently
preferred that the OPGL polypeptide discussed above is a human
10  OPGL polypeptide. In this embodiment, it is especially pre-
ferred that the human OPGL polypeptide has been modified by
substituting at least one amino acid sequence in SEQ ID NO: 2
(or in a polypeptide where Cys-221 in SEQ ID NO: 2 has been
substituted with serine) with at least one amino acid sequence
15  of equal or different length and containing a foreign $T_H$ epi-
tope. The substituted amino acid residues are selected from
residues 257-262, 289-303 and 222-243 in SEQ ID NO: 2. The
rationale behind such constructs is discussed in detail in the
examples.

20  Formulation of OPGL and modified OPGL polypeptides

When effecting presentation of the OPGL polypeptide or the
modified OPGL polypeptide to an animal's immune system by
means of administration thereof to the animal, the formulation
of the polypeptide follows the principles generally acknow-
25  ledged in the art.

Preparation of vaccines which contain peptide sequences as
active ingredients is generally well understood in the art, as
exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231;
4,599,230; 4,596,792; and 4,578,770, all incorporated herein
30  by reference. Typically, such vaccines are prepared as
injectables either as liquid solutions or suspensions; solid
forms suitable for solution in, or suspension in, liquid prior

to injection may also be prepared. The preparation may also be
emulsified. The active immunogenic ingredient is often mixed
with excipients which are pharmaceutically acceptable and
compatible with the active ingredient. Suitable excipients

5  are, for example, water, saline, dextrose, glycerol, ethanol,
or the like, and combinations thereof. In addition, if de-  —
sired, the vaccine may contain minor amounts of auxiliary
substances such as wetting or emulsifying agents, pH buffering
agents, or adjuvants which enhance the effectiveness of the

10 vaccines; cf. the detailed discussion of adjuvants below.


The vaccines are conventionally administered parenterally, by
injection, for example, either subcutaneously, intracutaneous-
ly, intradermally, subdermally or intramuscularly. Additional
formulations which are suitable for other modes of administra-

15 tion include suppositories and, in some cases, oral, buccal,
sublinqual, intraperitoneal, intravaginal, anal, epidural,
spinal, and intracranial formulations. For suppositories,
traditional binders and carriers may include, for example,
polyalkalene glycols or triglycerides; such suppositories may

20 be formed from mixtures containing the active ingredient in
the range of 0.5% to 10%, preferably 1-2%. Oral formulations
include such normally employed excipients as, for example,
pharmaceutical grades of mannitol, lactose, starch, magnesium
stearate, sodium saccharine, cellulose, magnesium carbonate,

25 and the like. These compositions take the form of solutions,
suspensions, tablets, pills, capsules, sustained release
formulations or powders and contain 10-95% of active ingredi-
ent, preferably 25-70%. For oral formulations, cholera toxin
is an interesting formulation partner (and also a possible

30 conjugation partner).


The polypeptides may be formulated into the vaccine as neutral
or salt forms. Pharmaceutically acceptable salts include acid
addition salts (formed with the free amino groups of the
peptide) and which are formed with inorganic acids such as,

for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium,

5 ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeuti-

10 cally effective and immunogenic. The quantity to be adminis-tered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Sui-table dosage ranges are of the order of several hundred micro-

15 grams active ingredient per vaccination with a preferred range from about 0.1 µg to 2,000 µg (even though higher amounts in the 1-10 mg range are contemplated), such as in the range from about 0.5 µg to 1,000 µg, preferably in the range from 1 µg to 500 µg and especially in the range from about 10 µg to 100 µg.

20 Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administra-tion followed by subsequent inoculations or other administra-tions.

The manner of application may be varied widely. Any of the

25 conventional methods for administration of a vaccine are applicable. These include oral application on a solid physio-logically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and

30 will vary according to the age of the person to be vaccinated and the formulation of the antigen.

Some of the polypeptides of the vaccine are sufficiently immunogenic in a vaccine, but for some of the others the

immune response will be enhanced if the vaccine further com-
prises an adjuvant substance.

Various methods of achieving adjuvant effect for the vaccine
are known. General principles and methods are detailed in "The
5  Theory and Practical Application of Adjuvants", 1995, Duncan
E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-
95170-6, and also in "Vaccines: New Generationn Immunological
Adjuvants", 1995, Gregoriadis G *et al.* (eds.), Plenum Press,
New York, ISBN 0-306-45283-9, both of which are hereby incor-
10 porated by reference herein.

It is especially preferred to use an adjuvant which can be
demonstrated to facilitate breaking of the autotolerance to
autoantigens; in fact, this is essential in cases where unmo-
dified OPGL is used as the active ingredient in the autovac-
15 cine. Non-limiting examples of suitable adjuvants are selected
from the group consisting of an immune targeting adjuvant; an
immune modulating adjuvant such as a toxin, a cytokine, and a
mycobacterial derivative; an oil formulation; a polymer; a
micelle forming adjuvant; a saponin; an immunostimulating
20 complex matrix (ISCOM matrix); a particle; DDA; aluminium
adjuvants; DNA adjuvants; γ-inulin; and an encapsulating
adjuvant. In general it should be noted that the disclosures
above which relate to compounds and agents useful as first,
second and third moieties in the analogues also refer *mutatis*
25 *mutandis* to their use in the adjuvant of a vaccine of the
invention.

The application of adjuvants include use of agents such as
aluminum hydroxide or phosphate (alum), commonly used as 0.05
to 0.1 percent solution in buffered saline, admixture with
30 synthetic polymers of sugars (e.g. Carbopol®) used as 0.25
percent solution, aggregation of the protein in the vaccine by
heat treatment with temperatures ranging between 70° to 101°C

for 30 second to 2 minute periods respectively and also aggre-
gation by means of cross-linking agents are possible. Aggrega-
tion by reactivation with pepsin treated antibodies (Fab
fragments) to albumin, mixture with bacterial cells such as *C.*
5   *parvum* or endotoxins or lipopolysaccharide components of gram-
negative bacteria, emulsion in physiologically acceptable oil
vehicles such as mannide mono-oleate (Aracel A) or emulsion
with 20 percent solution of a perfluorocarbon (Fluosol-DA)
used as a block substitute may also be employed. Admixture
10  with oils such as squalene and IFA is also preferred.

According to the invention DDA (dimethyldioctadecylammonium
bromide) is an interesting candidate for an adjuvant as is DNA
and γ-inulin, but also Freund's complete and incomplete adju-
vants as well as *quillaja* saponins such as QuilA and QS21 are
15  interesting as is RIBI. Further possibilities are monophos-
phoryl lipid A (MPL), the above mentioned C3 and C3d, and
muramyl dipeptide (MDP).

Liposome formulations are also known to confer adjuvant ef-
fects, and therefore liposome adjuvants are preferred accor-
20  ding to the invention.

Also immunostimulating complex matrix type (ISCOM® matrix)
adjuvants are preferred choices according to the invention,
especially since it has been shown that this type of adjuvants
are capable of up-regulating MHC Class II expression by APCs.
25  An ISCOM® matrix consists of (optionally fractionated) sapo-
nins (triterpenoids) from *Quillaja saponaria*, cholesterol, and
phospholipid. When admixed with the immunogenic protein, the
resulting particulate formulation is what is known as an ISCOM
particle where the saponin constitutes 60-70% w/w, the choles-
30  terol and phospholipid 10-15% w/w, and the protein 10-15% w/w.
Details relating to composition and use of immunostimulating
complexes can e.g. be found in the above-mentioned text-books

dealing with adjuvants, but also Morein B et al., 1995, Clin.
Immunother. 3: 461-475 as well as Barr IG and Mitchell GF,
1996, Immunol. and Cell Biol. 74: 8-25 (both incorporated by
reference herein) provide useful instructions for the prepara-
5  tion of complete immunostimulating complexes.

Another highly interesting (and thus, preferred) possibility
of achieving adjuvant effect is to employ the technique de-
scribed in Gosselin et al., 1992 (which is hereby incorporated
by reference herein). In brief, the presentation of a relevant
10  antigen such as an antigen of the present invention can be
enhanced by conjugating the antigen to antibodies (or antigen
binding antibody fragments) against the Fcγ receptors on
monocytes/macrophages. Especially conjugates between antigen
and anti-FcγRI have been demonstrated to enhance immunogenici-
15  ty for the purposes of vaccination.

Other possibilities involve the use of the targeting and
immune modulating substances (i.a. cytokines) mentioned above
as candidates for the first and second moieties in the modi-
fied versions of OPGL. In this connection, also synthetic
20  inducers of cytokines like poly I:C are possibilities.

Suitable mycobacterial derivatives are selected from the group
consisting of muramyl dipeptide, complete Freund's adjuvant,
RIBI, and a diester of trehalose such as TDM and TDE.

Suitable immune targeting adjuvants are selected from the
25  group consisting of CD40 ligand and CD40 antibodies or specif-
ically binding fragments thereof (cf. the discussion above),
mannose, a Fab fragment, and CTLA-4.

Suitable polymer adjuvants are selected from the group con-
sisting of a carbohydrate such as dextran, PEG, starch, man-

nan, and mannose; a plastic polymer such as; and latex such as
latex beads.

Yet another interesting way of modulating an immune response
is to include the OPGL immunogen (optionally together with
5  adjuvants and pharmaceutically acceptable carriers and vehic-
les) in a "virtual lymph node" (VLN) (a proprietary medical
device developed by ImmunoTherapy, Inc., 360 Lexington Avenue,
New York, NY 10017-6501). The VLN (a thin tubular device)
mimics the structrue and function of a lymph node. Insertion
10 of a VLN under the skin creates a site of sterile inflammation
with an upsurge of cytokines and chemokines. T- and B-cells as
well as APCs rapidly respond to the danger signals, home to
the inflamed site and accumulate inside the porous matrix of
the VLN. It has been shown that the necessary antigen dose
15 required to mount an immune response to an antigen is reduced
when using the VLN and that immune protection conferred by
vaccination using a VLN surpassed conventional immunization
using Ribi as an adjuvant. The technology is *i.a.* described
briefly in Gelber C *et al.*, 1998, "Elicitation of Robust
20 Cellular and Humoral Immune Responses to Small Amounts of
Immunogens Using a Novel Medical Device Designated the Virtual
Lymph Node", in: "From the Laboratory to the Clinic, Book of
Abstracts, October 12th – 15th 1998, Seascape Resort, Aptos,
California".

25 It is expected that the vaccine should be administered 1-6
times per year, such as 1, 2, 3, 4, 5, or 6 times a year to an
individual in need thereof. It has previously been shown that
the memory immunity induced by the use of the preferred
autovaccines according to the invention is not permanent, and
30 therefore the immune system needs to be periodically chal-
lenged with the OPGL or modified OPGL polypeptides.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise several different polypeptides in order to increase the immune
5 response, cf. also the discussion above concerning the choice of foreign T-cell epitope introductions. The vaccine may comprise two or more polypeptides, where all of the polypeptides are as defined above.

The vaccine may consequently comprise 3-20 different modified
10 or unmodified polypeptides, such as 3-10 different polypeptides.

Nucleic acid vaccination

As an alternative to classic administration of a peptide-based vaccine, the technology of nucleic acid vaccination (also
15 known as "nucleic acid immunisation", "genetic immunisation", and "gene immunisation") offers a number of attractive features.

First, in contrast to the traditional vaccine approach, nucleic acid vaccination does not require resource consuming
20 large-scale production of the immunogenic agent (e.g. in the form of industrial scale fermentation of microorganisms producing modified OPGL). Furthermore, there is no need to device purification and refolding schemes for the immunogen. And finally, since nucleic acid vaccination relies on the biochem-
25 ical apparatus of the vaccinated individual in order to produce the expression product of the nucleic acid introduced, the optimum posttranslational processing of the expression product is expected to occur; this is especially important in the case of autovaccination, since, as mentioned above, a
30 significant fraction of the original OPGL B-cell epitopes should be preserved in the modified molecule, and since B-cell epitopes in principle can be constituted by parts of any

(bio)molecule (e.g. carbohydrate, lipid, protein etc.). There-
fore, native glycosylation and lipidation patterns of the
immunogen may very well be of importance for the overall
immunogenicity and this is best ensured by having the host
5  producing the immunogen.

Hence, a preferred embodiment of the invention comprises
effecting presentation of modified OPGL to the immune system
by introducing nucleic acid(s) encoding the modified OPGL into
the animal's cells and thereby obtaining *in vivo* expression by
10  the cells of the nucleic acid(s) introduced.

In this embodiment, the introduced nucleic acid is preferably
DNA which can be in the form of naked DNA, DNA formulated with
charged or uncharged lipids, DNA formulated in liposomes, DNA
included in a viral vector, DNA formulated with a
15  transfection-facilitating protein or polypeptide, DNA formu-
lated with a targeting protein or polypeptide, DNA formulated
with Calcium precipitating agents, DNA coupled to an inert
carrier molecule, DNA encapsulated in chitin or chitosan, and
DNA formulated with an adjuvant. In this context it is noted
20  that practically all considerations pertaining to the use of
adjuvants in traditional vaccine formulation apply for the
formulation of DNA vaccines. Hence, all disclosures herein
which relate to use of adjuvants in the context of polypeptide
based vaccines apply *mutatis mutandis* to their use in nucleic
25  acid vaccination technology.

As for routes of administration and administration schemes of
polypeptide based vaccines which have been detailed above,
these are also applicable for the nucleic acid vaccines of the
invention and all discussions above pertaining to routes of
30  administration and administration schemes for polypeptides
apply *mutatis mutandis* to nucleic acids. To this should be
added that nucleic acid vaccines can suitably be administered

intraveneously and intraarterially. Furthermore, it is well-
known in the art that nucleic acid vaccines can be adminis-
tered by use of a so-called gene gun, and hence also this and
equivalent modes of administration are regarded as part of the
5   present invention. Finally, also the use of a VLN in the
administration of nucleic acids has been reported to yield ‾
good results, and therefore this particular mode of admini-
stration is particularly preferred.

Furthermore, the nucleic acid(s) used as an immunization agent
10  can contain regions encoding the 1$^{st}$, 2$^{nd}$ and/or 3$^{rd}$ moieties,
e.g. in the form of the immunomodulating substances described
above such as the cytokines discussed as useful adjuvants. A
preferred version of this embodiment encompasses having the
coding region for the analogue and the coding region for the
15  immunomodulator in different reading frames or at least under
the control of different promoters. Thereby it is avoided that
the analogue or epitope is produced as a fusion partner to the
immunomodulator. Alternatively, two distinct nucleotide frag-
ments can be used, but this is less preferred because of the
20  advantage of ensured co-expression when having both coding
regions included in the same molecule.

Accordingly, the invention also relates to a composition for
inducing production of antibodies against OPGL, the composi-
tion comprising
25  -    a nucleic acid fragment or a vector of the invention (cf.
the discussion of vectors below), and
-    a pharmaceutically and immunologically acceptable vehicle
and/or carrier and/or adjuvant as discussed above.

Under normal circumstances, the OPGL variant-encoding nucleic
30  acid is introduced in the form of a vector wherein expression
is under control of a viral promoter. For more detailed dis-
cussions of vectors according to the invention, cf. the dis-
cussion below. Also, detailed disclosures relating to the

formulation and use of nucleic acid vaccines are available, cf. Donnelly JJ *et al*, 1997, Annu. Rev. Immunol. **15:** 617-648 and Donnelly JJ *et al.*, 1997, Life Sciences **60:** 163-172. Both of these references are incorporated by reference herein.

5 Live vaccines

A third alternative for effecting presentation of modified OPGL to the immune system is the use of live vaccine technology. In live vaccination, presentation to the immune system is effected by administering, to the animal, a non-pathogenic

10 microorganism which has been transformed with a nucleic acid fragment encoding a modified OPGL or with a vector incorporating such a nucleic acid fragment. The non-pathogenic microorganism can be any suitable attenuated bacterial strain (attenuated by means of passaging or by means of removal of patho-

15 genic expression products by recombinant DNA technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic *Streptococcus* spp., *E. coli*, *Salmonella* spp., *Vibrio cholerae*, *Shigella*, etc. Reviews dealing with preparation of state-of-the-art live vaccines can e.g. be found in Saliou P, 1995, Rev. Prat. **45:**

20 1492-1496 and Walker PD, 1992, Vaccine **10:** 977-990, both incorporated by reference herein. For details about the nucleic acid fragments and vectors used in such live vaccines, cf. the discussion below.

As an alternative to bacterial live vaccines, the nucleic acid

25 fragment of the invention discussed below can be incorporated in a non-virulent viral vaccine vector such as a vaccinia strain or any other suitable pox virus.

Normally, the non-pathogenic microorganism or virus is administered only once to the animal, but in certain cases it may

30 be necessary to administer the microorganism more than once in a lifetime in order to maintain protective immunity. It is

even contemplated that immunization schemes as those detailed
above for polypeptide vaccination will be useful when using
live or virus vaccines.

Alternatively, live or virus vaccination is combined with
5  previous or subsequent polypeptide and/or nucleic acid vacci-
nation. For instance, it is possible to effect primary immuni-
zation with a live or virus vaccine followed by subsequent
booster immunizations using the polypeptide or nucleic acid
approach.

10 The microorganism or virus can be transformed with nucleic
acid(s) containing regions encoding the $1^{st}$, $2^{nd}$ and/or $3^{rd}$
moieties, e.g. in the form of the immunomodulating substances
described above such as the cytokines discussed as useful
adjuvants. A preferred version of this embodiment encompasses
15 having the coding region for the analogue and the coding
region for the immunomodulator in different reading frames or
at least under the control of different promoters. Thereby it
is avoided that the analogue or epitopes are produced as
fusion partners to the immunomodulator. Alternatively, two
20 distinct nucleotide fragments can be used as transforming
agents. Of course, having the $1^{st}$ and/or $2^{nd}$ and/or $3^{rd}$ moieties
in the same reading frame can provide as an expression pro-
duct, an analogue of the invention, and such an embodiment is
especially preferred according to the present invention.

25 <u>Use of the method of the invention in disease treatment</u>

As will be appreciated from the discussions above, the provi-
sion of the method of the invention allows for control of
diseases characterized by excessive loss of bone mass. In this
context, the disease osteoporosis is the key target for the
30 inventive method but also bone loss associated with compli-
cated bone fractures is a feasible target for treatment/ame-

lioration. Hence, an important embodiment of the method of the
invention for down-regulating OPGL activity comprises treating
and/or preventing and/or ameliorating osteoporosis or other
conditions characterized by excess bone resorption, the method
5  comprising down-regulating OPGL activity according to the
method of the invention to such an extent that the rate of  —
bone resorption is significantly decreased.

In the present context such a significant decrease in bone
resorption is at least 3% compared to the pathological rate,
10  but higher percentages are contemplated, such as at least 5%,
at least 7%, at least 9%, at least 11%, at least 13%, at least
15%, and at least 17%, but even higher percentages are ex-
pected, such as at least 20%, or even at least 30%. It is
especially preferred that the decrease in bone resorption
15  results in an inversion of the balance between bone formation
and bone resorption, i.e. that the rate of bone formation is
brought to exceed the rate of bone resorption. Of course, this
imbalance should not be maintained (since it would result in
osteopetrosis), but by carefully controlling the number and
20  immunological impact of immunizations of the individual in
need thereof it is possible to obtain a balance over time
which results in a net conservation of bone mass. Alterna-
tively, if in an individual the method of the invention cannot
terminate bone loss, the method of the invention can (option-
25  ally in combination with other known methods for reducing bone
loss in osteoporosis patients) be used to obtain a significant
reduction in bone loss, thereby prolonging the time where
sufficient bone mass is present in the individual.

Methods for measuring the rate of bone resorption and bone
30  formation are known in the art. It is by means of biochemical
assays possible to gauge the rate of bone resorption by
measuring the blood concentration of certain fragments of
collagen type I (cf. WO 93/15107 and WO 94/14844). Alterna-

42

tively, the rate of bone loss can be assessed by physical means; representative disclosures in the art of methods for assessing bone mass by non-invasive, physical methods can be found in WO 88/06862, WO 94/12855, WO 95/14431, and WO

5 97/00643.

Peptides, polypeptides, and compositions of the invention

As will be apparent from the above, the present invention is based on the concept of immunising individuals against the OPGL antigen in order to indirectly obtain a reduced

10 osteoclast activity. The preferred way of obtaining such an immunization is to use modified versions of OPGL, thereby providing molecules which have not previously been disclosed in the art.

It is believed that the modified OPGL molecules discussed

15 herein are inventive in their own right, and therefore an important part of the invention pertains to an OPGL analogue which is derived from an animal OPGL wherein is introduced a modification which has as a result that immunization of the animal with the analogue induces production of antibodies

20 reacting specifically with the unmodified OPGL polypeptide. Preferably, the nature of the modification conforms with the types of modifications described above when discussing various embodiments of the method of the invention when using modified OPGL. Hence, any disclosure presented herein pertaining to

25 modified OPGL molecules are relevant for the purpose of describing the OPGL analogues of the invention, and any such disclosures apply *mutatis mutandis* to the description of these analogues.

It should be noted that preferred modified OPGL molecules com-

30 prises modifications which results in a polypeptide having a sequence identity of at least 70% with OPGL or with a

subsequence thereof of at least 10 amino acids in length.
Higher sequence identities are preferred, e.g. at least 75% or
even at least 80, 85, 90, or 95%. The sequence identity for
proteins and nucleic acids can be calculated as $(N_{ref}$ –

5   $N_{dif}) \cdot 100/N_{ref}$, wherein $N_{dif}$ is the total number of non-identical
residues in the two sequences when aligned and wherein $N_{ref}$ is
the number of residues in one of the sequences. Hence, the DNA
sequence AGTCAGTC will have a sequence identity of 75% with
the sequence AATCAATC ($N_{dif}$=2 and $N_{ref}$=8).


10  The invention also pertains to compositions useful in
exercising the method of the invention. Hence, the invention
also relates to an immunogenic composition comprising an
immunogenically effective amount of an OPGL polypeptide which
is a self-protein in an animal, said OPGL polypeptide being

15  formulated together with an immunologically acceptable
adjuvant so as to break the animal's autotolerance towards the
OPGL polypeptide, the composition further comprising a
pharmaceutically and immunologically acceptable diluent and/or
vehicle and/or carrier and/or excipient. In other words, this

20  part of the invention pertains to the formulations of
naturally occurring OPGL polypeptides which have been
described in connection with embodiments of the method of the
invention.


The invention also relates to an immunogenic composition com-
25  prising an immunologically effective amount of an OPGL
analogue defined above, said composition further comprising a
pharmaceutically and immunologically acceptable diluent and/or
vehicle and/or carrier and/or excipient and optionally an
adjuvant. In other words, this part of the invention concerns
30  formulations of modified OPGL, essentially as described above.
The choice of adjuvants, carriers, and vehicles is accordingly
in line with what has been discussed above when referring to

formulation of modified and unmodified OPGL for use in the
inventive method for the down-regulation of OPGL.


The polypeptides are prepared according to methods well-known
in the art. Longer polypeptides are normally prepared by means
5 of recombinant gene technology including introduction of a —
nucleic acid sequence encoding the OPGL analogue into a sui-
table vector, transformation of a suitable host cell with the
vector, expression of the nucleic acid sequence, recovery of
the expression product from the host cells or their culture
10 supernatant, and subseqeunt purification and optional further
modification, e.g. refolding or derivatization.


Shorter peptides are preferably prepared by means of the well-
known techniques of solid- or liquid-phase peptide synthesis.
However, recent advances in this technology has rendered
15 possible the production of full-length polypeptides and pro-
teins by these means, and therefore it is also within the
scope of the present invention to prepare the long constructs
by synthetic means.


Nucleic acid fragments and vectors of the invention


20 It will be appreciated from the above disclosure that modified
OPGL polypeptides can be prepared by means of recombinant gene
technology but also by means of chemical synthesis or
semisynthesis; the latter two options are especially relevant
when the modification consists in coupling to protein carriers
25 (such as KLH, diphtheria toxoid, tetanus toxoid, and BSA) and
non-proteinaceous molecules such as carbohydrate polymers and
of course also when the modification comprises addition of
side chains or side groups to an OPGL polypeptide-derived
peptide chain.


30 For the purpose of recombinant gene technology, and of course
also for the purpose of nucleic acid immunization, nucleic

acid fragments encoding modified OPGL are important chemical
products. Hence, an important part of the invention pertains
to a nucleic acid fragment which encodes an OPGL analogue,
*i.e.* an OPGL derived polypeptide which either comprises the
5  natural OPGL sequence to which has been added or inserted a
fusion partner or, preferably an OPGL derived polypeptide   —
wherein has been introduced a foreign T-cell epitope by means
of insertion and/or addition, preferably by means of substitu-
tion and/or deletion. The nucleic acid fragments of the inven-
10  tion are either DNA or RNA fragments.

The nucleic acid fragments of the invention will normally be
inserted in suitable vectors to form cloning or expression
vectors carrying the nucleic acid fragments of the invention;
such novel vectors are also part of the invention. Details
15  concerning the construction of these vectors of the invention
will be discussed in context of transformed cells and microor-
ganisms below. The vectors can, depending on purpose and type
of application, be in the form of plasmids, phages, cosmids,
mini-chromosomes, or virus, but also naked DNA which is only
20  expressed transiently in certain cells is an important vector.
Preferred cloning and expression vectors of the invention are
capable of autonomous replication, thereby enabling high copy-
numbers for the purposes of high-level expression or high-
level replication for subsequent cloning.

25  The general outline of a vector of the invention comprises the
following features in the 5'-3' direction and in operable
linkage: a promoter for driving expression of the nucleic acid
fragment of the invention, optionally a nucleic acid sequence
encoding a leader peptide enabling secretion (to the
30  extracellular phase or, where applicable, into the periplasma)
of or integration into the membrane of the polypeptide frag-
ment, the nucleic acid fragment of the invention, and option-
ally a nucleic acid sequence encoding a terminator. When

operating with expression vectors in producer strains or cell-
lines it is for the purposes of genetic stability of the
transformed cell preferred that the vector when introduced
into a host cell is integrated in the host cell genome. In
5   contrast, when working with vectors to be used for effecting
*in vivo* expression in an animal (*i.e.* when using the vector in
DNA vaccination) it is for security reasons preferred that the
vector is incapable of being integrated in the host cell
genome; typically, naked DNA or non-integrating viral vectors
10  are used, the choices of which are well-known to the person
skilled in the art

The vectors of the invention are used to transform host cells
to produce the modified OPGL polypeptide of the invention.
Such transformed cells, which are also part of the invention,
15  can be cultured cells or cell lines used for propagation of
the nucleic acid fragments and vectors of the invention, or
used for recombinant production of the modified OPGL polypep-
tides of the invention. Alternatively, the transformed cells
can be suitable live vaccine strains wherein the nucleic acid
20  fragment (one single or multiple copies) have been inserted so
as to effect secretion or integration into the bacterial
membrane or cell-wall of the modified OPGL.

Preferred transformed cells of the invention are microorga-
nisms such as bacteria (such as the species *Escherichia* [e.g.
25  *E.coli*], *Bacillus* [e.g. *Bacillus subtilis*], *Salmonella*, or
*Mycobacterium* [preferably non-pathogenic, e.g. *M. bovis* BCG]),
yeasts (such as *Saccharomyces cerevisiae*), and protozoans.
Alternatively, the transformed cells are derived from a
multicellular organism such as a fungus, an insect cell, a
30  plant cell, or a mammalian cell. Most preferred are cells
derived from a human being, cf. the discussion of cell lines
and vectors below. Recent results have shown great promise in
the use of a commercially available *Drosophila melanogaster*

cell line (the Schneider 2 (S₂) cell line and vector system
available from Invitrogen) for the recombinant production of
polypeptides in applicants' lab, and therefore this expression
system is particularly preferred.

5    For the purposes of cloning and/or optimized expression it is
preferred that the transformed cell is capable of replicating
the nucleic acid fragment of the invention. Cells expressing
the nucleic fragment are preferred useful embodiments of the
invention; they can be used for small-scale or large-scale
10   preparation of the modified OPGL or, in the case of non-patho-
genic bacteria, as vaccine constituents in a live vaccine.

When producing the modified OPGL of the invention by means of
transformed cells, it is convenient, although far from essen-
tial, that the expression product is either exported out into
15   the culture medium or carried on the surface of the trans-
formed cell.

When an effective producer cell has been identified it is pre-
ferred, on the basis thereof, to establish a stable cell line
which carries the vector of the invention and which expresses
20   the nucleic acid fragment encoding the modified OPGL. Prefer-
ably, this stable cell line secretes or carries the OPGL ana-
logue of the invention, thereby facilitating purification
thereof.

In general, plasmid vectors containing replicon and control
25   sequences which are derived from species compatible with the
host cell are used in connection with the hosts. The vector
ordinarily carries a replication site, as well as marking se-
quences which are capable of providing phenotypic selection in
transformed cells. For example, *E. coli* is typically trans-
30   formed using pBR322, a plasmid derived from an *E. coli* species
(see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains

genes for ampicillin and tetracycline resistance and thus
provides easy means for identifying transformed cells. The pBR
plasmid, or other microbial plasmid or phage must also con-
tain, or be modified to contain, promoters which can be used
5  by the prokaryotic microorganism for expression.

Those promoters most commonly used in recombinant DNA con-
struction include the B-lactamase (penicillinase) and lactose
promoter systems (Chang et al., 1978; Itakura et al., 1977;
Goeddel et al., 1979) and a tryptophan (trp) promoter system
10 (Goeddel et al., 1979; EP-A-0 036 776). While these are the
most commonly used, other microbial promoters have been dis-
covered and utilized, and details concerning their nucleotide
sequences have been published, enabling a skilled worker to
ligate them functionally with plasmid vectors (Siebwenlist et
15 al., 1980). Certain genes from prokaryotes may be expressed
efficiently in *E. coli* from their own promoter sequences,
precluding the need for addition of another promoter by arti-
ficial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast
20 cultures may also be used, and here the promoter should be
capable of driving expression. *Saccharomyces cerevisiase*, or
common baker's yeast is the most commonly used among eukaryo-
tic microorganisms, although a number of other strains are
commonly available. For expression in *Saccharomyces*, the
25 plasmid YRp7, for example, is commonly used (Stinchcomb et
al., 1979; Kingsman et al., 1979; Tschemper et al., 1980).
This plasmid already contains the trpl gene which provides a
selection marker for a mutant strain of yeast lacking the
ability to grow in tryptophan for example ATCC No. 44076 or
30 PEP4-1 (Jones, 1977). The presence of the trpl lesion as a
characteristic of the yeast host cell genome then provides an
effective environment for detecting transformation by growth
in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the pro-
moters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or
other glycolytic enzymes (Hess et al., 1968; Holland et al.,
1978), such as enolase, glyceraldehyde-3-phosphate

5 dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-
fructokinase, glucose-6-phosphate isomerase, 3-phosphoglyce-
rate mutase, pyruvate kinase, triosephosphate isomerase,
phosphoglucose isomerase, and glucokinase. In constructing
suitable expression plasmids, the termination sequences asso-

10 ciated with these genes are also ligated into the expression
vector 3' of the sequence desired to be expressed to provide
polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of tran-
scription controlled by growth conditions are the promoter

15 region for alcohol dehydrogenase 2, isocytochrome C, acid
phosphatase, degradative enzymes associated with nitrogen
metabolism, and the aforementioned glyceraldehyde-3-phosphate
dehydrogenase, and enzymes responsible for maltose and
galactose utilization. Any plasmid vector containing a yeast-

20 compatible promoter, origin of replication and termination
sequences is suitable.

In addition to microorganisms, cultures of cells derived from
multicellular organisms may also be used as hosts. In princip-
le, any such cell culture is workable, whether from vertebrate

25 or invertebrate culture. However, interest has been greatest
in vertebrate cells, and propagation of vertebrate in culture
(tissue culture) has become a routine procedure in recent
years (Tissue Culture, 1973). Examples of such useful host
cell lines are VERO and HeLa cells, Chinese hamster ovary

30 (CHO) cell lines, and W138, BHK, COS-7 293, *Spodoptera
frugiperda* (SF) cells (commercially available as complete
expression systems from *i.a.* Protein Sciences, 1000 Research
Parkway, Meriden, CT 06450, U.S.A. and from Invitrogen), and

MDCK cell lines. In the present invention, an especially preferred cell line is $S_2$ available from Invitrogen, PO Box 2312, 9704 CH Groningen, The Netherlands.

Expression vectors for such cells ordinarily include (if
5  necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the
10 expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment
15 which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *Hind*III site toward the *Bgl*I site located in the viral origin of replication. Further, it
20 is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construc-
25 tion of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

## Identification of useful OPGL analogues

It will be clear to the skilled person that not all possible
variants or modifications of native OPGL will have the ability
to elicit antibodies in an animal which are cross-reactive
with the native form. It is, however, not difficult to set up
5  an effective standard screen for modified OPGL molecules which
fulfill the minimum requirements for immunological reactivity
discussed herein. Hence, another part of the invention con-
cerns a method for the identification of a modified OPGL
polypeptide which is capable of inducing antibodies against
10 unmodified OPGL in an animal species where the unmodified OPGL
polypeptide is a (non-immunogenic) self-protein, the method
comprising

-       preparing, by means of peptide synthesis or genetic engi-
15      neering techniques, a set of mutually distinct modified
        OPGL polypeptides wherein amino acids have been added to,
        inserted in, deleted from, or substituted into the amino
        acid sequence of an OPGL polypeptide of the animal spe-
        cies thereby giving rise to amino acid sequences in the
20      set which comprise T-cell epitopes which are foreign to
        the animal species, or preparing a set of nucleic acid
        fragments encoding the set of mutually distinct modified
        OPGL polypeptides,
-       testing members of the set of modified OPGL polypeptides
25      or nucleic acid fragments for their ability to induce
        production of antibodies by the animal species against
        the unmodified OPGL, and
-       identifying and optionally isolating the member(s) of the
        set of modified OPGL polypeptides which significantly
30      induces antibody production against unmodified OPGL in
        the species or identifying and optionally isolating the
        polypeptide expression products encoded by members of the
        set of nucleic acid fragments which significantly induces

antibody production against unmodified OPGL in the animal
species.

In this context, the "set of mutually distinct modified OPGL
polypeptides" is a collection of non-identical modified OPGL
5 polypeptides which have e.g. been selected on the basis of the
criteria discussed above (e.g. in combination with studies of
circular dichroism, NMR spectra, and/or X-ray diffraction pat-
terns). The set may consist of only a few members but it is
contemplated that the set may contain several hundred members.

10 The test of members of the set can ultimately be performed *in
vivo*, but a number of *in vitro* tests can be applied which
narrow down the number of modified molecules which will serve
the purpose of the invention.

Since the goal of introducing the foreign T-cell epitopes is
15 to support the B-cell response by T-cell help, a prerequisite
is that T-cell proliferation is induced by the modified OPGL.
T-cell proliferation can be tested by standardized prolifera-
tion assays *in vitro*. In short, a sample enriched for T-cells
is obtained from a subject and subsequently kept in culture.
20 The cultured T-cells are contacted with APCs of the subject
which have previously taken up the modified molecule and
processed it to present its T-cell epitopes. The proliferation
of T-cells is monitored and compared to a suitable control
(e.g. T-cells in culture contacted with APCs which have pro-
25 cessed intact, native OPGL). Alternatively, proliferation can
be measured by determining the concentration of relevant
cytokines released by the T-cells in response to their recog-
nition of foreign T-cells.

Having rendered highly probable that at least one modified
30 OPGL of either type of set is capable of inducing antibody
production against OPGL, it is possible to prepare an

immunogenic composition comprising at least one modified OPGL
polypeptide which is capable of inducing antibodies against
unmodified OPGL in an animal species where the unmodified OPGL
polypeptide is a self-protein, the method comprising admixing
5  the member(s) of the set which significantly induces produc-
tion of antibodies in the animal species which are reactive
with OPGL with a pharmaceutically and immunologically accept-
able carrier and/or vehicle and/or diluent and/or excipient,
optionally in combination with at least one pharmaceutically
10 and immunologically acceptable adjuvant.

The above aspects of the invention pertaining to test of
polypeptide sets are conveniently carried out by initially
preparing a number of mutually distinct nucleic acid sequences
or vectors of the invention, inserting these into appropriate
15 expression vectors, transforming suitable host cells with the
vectors, and expressing the nucleic acid sequences of the
invention. These steps can be followed by isolation of the
expression products. It is preferred that the nucleic acid
sequences and/or vectors are prepared by methods comprising
20 exercise of a molecular amplification technique such as PCR or
by means of nucleic acid synthesis.

Another part of the invention concerns a method for the treat-
ment, prophylaxis or amelioration of diseases characterized by
excess bone resorption in an animal, including a human being,
25 the method comprising administering, to the animal, an effec-
tive amount of at least one substance different from
osteoprotegerin which blocks the stimulatory effect of OPGL on
osteoclast activity. It is presently believed that such an
approach has never been suggested in the art.

30 The preferred embodiment of this part of the invention in-
volves use of an OPGL-specific antibody (poly- or monoclonal)
or a specifically binding variant thereof as the substance
blocking the stimulatory effect of OPGL. It is preferred that

the antibody is an IgG or IgM molecule, or that the specifi-
cally binding varian is derived from IgG or IgM. The specifi-
cally binding variant of the antibody can conveniently be a
Fab fragment, a F(ab')$_2$ fragment, a humanized monoclonal anti-
5 body or fragment thereof, or a di- or multimeric antibody
fragment such as a diabody (a bispecific and dimeric artifi-
cial antibody-derived molecule produced by Cambridge Antibody
Technology).


EXAMPLE


10 It has been decided to clone or synthesize cDNAs encoding
murine and human OPGL in the truncated version comprising
amino acid residues 158-316 in the murine case and residues
159-317 in the human case (numbers correspond to the numbering
in SEQ ID NOs: 2 and 4, respectively). As these truncated
15 versions exhibit biological activity, it is logical to direct
the autoantibodies against this part of OPGL. In addition, it
makes the proteins smaller and thus easier to handle.


A synthetic cDNA encoding the murine OPGL residues 158-316 has
been synthesized removing sub-optimal *Eschericia coli* and
20 *Pichia pastoris* codons from the published sequence. Addition-
ally, an N-terminal Histidine tag, part of the cleavage site
of the alpha mating factor signal sequence from *Sacharomyces
cerevisiae*, and suitable restriction enzymes have been incor-
porated into the open reading frame (cf. SEQ ID NO: 7).


25 This cDNA encoding wild type murine OPGL has been cloned into
a standard *Eschericia coli* expression vector (pTrc99a) using
*Bsp*HI and *Hind*III restriction enzymes and a standard cloning
vector (pBluescript KS+) using *Sac*I and *Kpn*I restriction
enzymes (yielding SEQ ID NO: 9).

Expression in *Eschericia coli* cells resulted in approximately
30% recombinant OPGL of the total *Eschericia coli* protein. The
protein has been refolded and purified using the following
procedure:

5  1.  Cells are harvested by centrifugation.

    2.  Cells are resuspended in phosphate buffered saline (PBS)
       and recentrifuged.

    3.  The supernatant is discarded and the cells are resuspen-
       ded in three volumes (100 mM Tris[hydroxymethyl]aminome-

10      thane hydrochloride, 5 mM dithiotreitol (DTT), 0.5 M
       NaCl, pH 8.0).

    4.  The cells are added 8 µl 50 mM PMSF and 80 µl lysozyme
       (10 mg/ml) per gram cell and incubated at room tempera-
       ture for 20 min.

15  5.  For each gram cell pellet, 4 mg deoxychloric acid is
       added, and the suspension is incubated at 37°C until it
       appears viscous.

    6.  20 µl DNase (1 mg/ml) pr. gram cell weight is added, and
       $MgCl_2$ to 5 mM, and the suspension is incubated at room

20      temperature for 30 min.

    7.  The suspension is sonicated on ice until the viscosity
       disappears.

    8.  After centrifugation (20000 x g for 30 min) the pellet is
       resuspended in $H_2O$, recentrifuged and resuspended in 3 ml

25      1 M urea per gram cell weight.

    9.  After centrifugation (20000 x g for  30 min) the pellet
       is resuspended in 1 M Guanidine hydrochloride, 100 mM
       Tris[hydroxymethyl]aminomethane hydrochloride, pH 7.5.

    10. After centrifugation (20000 x g for 30 min) the pellet is

30      resuspended in 6 M Guanidine hydrochloride, 20 mM
       Tris[hydroxymethyl]aminomethane hydrochloride, 5% etha-
       nol, 1% beta-mercaptoethanol, pH 8.0, and stirred at 4°C
       overnight.

11.  After centrifugation (40000 x g for 1-4 hours) the
     supernatant is filtered and stored at -20°C.
12.  The solubilized inclusion bodies are separated by gel
     filtration chromatography using Superdex 200 material
5    (Pharmacia).
13.  The fractions containing the recombinant OPGL are pooled
     and diluted to 0.1 mg/ml with 1,5M Guanidine hydrochlo-
     ride, 20 mM Tris[hydroxymethyl]aminomethane hydrochlo-
     ride, 1 mM DTT, pH 7.5.
10  14.  The material is dialyzed overnight at 4°C against 10
     volumes 1,5M Guanidine hydrochloride, 20 mM
     Tris[hydroxymethyl]aminomethane hydrochloride, 1 mM DTT,
     pH 7.5
15.  The material is dialyzed overnight at 4°C against 10
15   volumes 1,0 M Guanidine hydrochloride, 20 mM
     Tris[hydroxymethyl]aminomethane hydrochloride, 1 mM DTT,
     pH 7.5
16.  The material is dialyzed overnight at 4°C against 10
     volumes 0,5 M Guanidine hydrochloride, 20 mM
20   Tris[hydroxymethyl]aminomethane hydrochloride, 1 mM DTT,
     pH 7.5
17.  The material is dialyzed overnight at 4°C against 10
     volumes 20 mM Tris[hydroxymethyl]aminomethane hydrochlo-
     ride, 150 mM Arginine, 1 mM DTT, pH 7.5
25  18.  The material is dialyzed overnight at 4°C against 10
     volumes 20 mM Tris[hydroxymethyl]aminomethane hydrochlo-
     ride, 150 mM Arginine, pH 7.5
19.  The refolded material is freeze dried and stored at
     -20°C.

30  The efficiency of refolding using this procedure is
approximately 40%, and the purity in excess of 65%. The
purification procedure and refolding process are still subject
to further improvements. Immobilized refolding are under
investigation, and enzymatic removal of the Histidine-tag will

be performed essentially as described by Pedersen *et al.*,
1999. The nature of the recombinant protein has been
characterized and verified using SDS-PAGE, N-terminal sequen-
cing, amino acid analysis, and mass spectrometry.

5   A cysteine substitution mutant of the wild type murine OPGL is
under construction (wherein a cysteine corresponding to amino
acid residue in SEQ ID NO: 4 is substituted with serine; cf.
SEQ ID NOs: 11 and 12). This is done to eliminate potential
stability problems with the purified recombinant protein. This
10  mutated OPGL truncate will serve as basis for vaccine con-
structs in complete analogy with the description below which
sets out from the DNA having SEQ ID NO: 9. Further, a corre-
sponding Cys-Ser mutant (where Cys-221 is substituted) of
human OPGL will also be produced for the same purposes.

15  The vaccine molecules are initially constructed by insertion
or in-substitution of either the P2 or P30 epitope from teta-
nus toxoid at selected positions. Other suitable immunodomi-
nant T-cell epitopes may be used at a later stage.

The selected positions for the introduction of variation are
20  chosen based on knowledge of existing or predicted B-cell
epitopes and predicted secondary structure elements of the
native molecule, as well as using alignments with the existing
three dimensional structures of TNFα (1a8m.pdb) and CD40
ligand (1aly.pdb) for modelling the secondary and tertiary
25  structure of the extracellular part of OPGL. The introduction
in the murine molecule will take place in areas corresponding
to amino acid residues 170-192, 198-218, 221-246, 256-261, and
285-316 (cf. the amino acid numbering in SEQ ID NO: 4), where-
as the introduction in the human molecule will take place in
30  areas corresponding to amino acid residues 171-193, 199-219,
222-247, 257-262, and 286-317.

Four variants of murine OPGL have by now been constructed and
expressed recombinantly in *Eschericia coli*:

**DNA encoding mOPGL[158-316]_P30[256-261] with an N-terminal
Histidine tag (SEQ ID NO: 13):**

5  PCR of SEQ ID NO: 9 was performed using SEQ ID NOs: 22 and 25
as primers. The resulting PCR fragment was restriction di-
gested with *SacII* and *KpnI* and subsequently purified from an
agarose gel. A second PCR using SEQ ID NO: 9 as template was
performed using primer SEQ ID NO: 26 and a vector specific

10  primer. The resulting PCR fragment was restriction digested
with *KpnI* and *HindIII*. Both fragments were then ligated to SEQ
ID NO: 9 in pBluescript KS+ restriction digested with *SacII*
and *HindIII*. To correct a single base mutation in this con-
struct, PCR using the construct as template was performed with

15  primers SEQ ID NOs: 33 and 29. The resulting PCR fragment was
restriction digested with *PstI* + *EcoRI*, gel purified and
subsequently ligated to the erroneous construct digested with
*PstI* and *EcoRI*. The verified construct (SEQ ID NO: 13) was
then transferred to pTrc99a using *BspHI* and *HindIII* restric-

20  tion enzymes.

**DNA encoding mOPGL[158-316]_P2[256-261] with an N-terminal
Histidine tag (SEQ ID NO: 15):**
PCR was performed using primers SEQ ID NOs: 27 and 28 without
template. The resulting PCR fragment was restriction digested

25  with *PstI* and *EcoRI* and subsequently purified from an agarose
gel. The resulting fragment was then ligated to SEQ ID NO: 9
in pBluescript KS+ restriction digested with *SacII* and
*HindIII*. The verified construct (SEQ ID NO: 15) was
subsequently transferred to pTrc99a using *BspHI* and *HindIII*

30  restriction enzymes

**DNA encoding mOPGL[158-316]_P2[288-302] with an N-terminal
Histidine tag (SEQ ID NO: 17):**

PCR of SEQ ID NO: 9 was performed using primers SEQ ID NOs: 22 and 29. The resulting PCR fragment was restriction digested with *Pst*I and *Bst*BI and subsequently purified from an agarose gel. A second PCR using SEQ ID NO: 9 as template was performed

5 using primer SEQ ID NO: 30 and a vector specific primer. The resulting PCR fragment was restriction digested with *Bst*BI and *Kpn*I and subsequently gel purified. Both fragments were then ligated to SEQ ID NO: 9 in pBluescript KS+ restriction digested with *Pst*I and *Kpn*I. The verified construct (SEQ ID NO:

10 17) was then transferred to pTrc99a using *Bsp*HI and *Hind*III restriction enzymes.


DNA encoding mOPGL[158-316]_P30[221-241] with an N-terminal Histidine tag (SEQ ID NO: 19):

PCR of SEQ ID NO: 9 was performed using primers SEQ ID NOs: 22

15 and 23. The resulting PCR fragment was restriction digested with *Sac*II and *Kpn*I and subsequently purified from an agarose gel. A second PCR using SEQ ID NO: 9 as template was performed using primer SEQ ID NOs: 24 and 31. The PCR fragment was restriction digested with *Kpn*I and *Eco*RI and subsequently gel

20 purified. Both fragments were then ligated to SEQ ID NO: 9 in pBluescript KS+ restriction digested with *Sac*II and *Eco*RI. The verified construct (SEQ ID NO: 19) was then transferred to pTrc99a using *Bsp*HI and *Hind*III restriction enzymes.


Expression of these truncated variants of OPGL have taken

25 place in *E. coli* yielding over 20% of total protein for all variants. A further development of the above-described purifi-cation and refolding procedure for the wild type protein (SEQ ID NO: 9) will be performed. This procedure will serve as a basis for the development of optimal procedures for each of

30 the variants. Immobilized refolding of the variant proteins utilizing the Histidine tag is another approach that is being pursued.

Alternatively, the variants can be directly transferred to
*Pichia pastoris* expression vectors using restriction enzymes,
or other yeast expression systems using PCR if glycosylation
is desired. It should be noted that the glycosylation is not
5  needed for biological activity *in vivo* of OPGL. It is also
possible to express the truncated OPGL in human 293 fibro-
blasts as reported in Lacey *et al*. Expression in insect cells
will also be considered (e.g. the Schneider 2 ($S_2$) cell line and
vector system or the *Spodoptera frugiperda* (SF) cell and
10  vector systems, both available from Invitrogen).

The purified variants will be used for antibody production in
rabbits for later use as detection tools as there exist no
commercially available antibodies. In addition, this material
will be a very valuable tool in the biological assays needed
15  to evaluate the autovaccine candidates. The preparation of the
antibodies will be performed using standard methods known in
the art.

Selecting the best autovaccine candidate is based on assess-
ment of inhibitory activity in *in vitro* assays for osteoclast
20  maturation/activation or in *in vivo* animal models for osteopo-
rosis. Useful assay and model systems are described in the
literature (e.g. in Lacey *et al*., Fuller *et al*., and Simonet
*et al*.).

The activity of the recombinant proteins will be analyzed by
25  intravenous injection of 100 µl into un-anaesthetized male
Balb/C mice (0, 0.1, and 1.0 mg protein pr. kg mouse) and one
hour later withdrawal of 125 µl blood from the major eye vein
using capillary tubes coated with calcium saturated heparin.
The calcium levels are measured using an ICA2 (Radiometer,
30  Denmark). Purified, and refolded recombinant murine OPGL (SEQ
ID NO: 9) is reactive in this assay, increasing the circula-
ting levels of ionized calcium by up to 10%.

The autovaccine candidates will e.g. be evaluated using autovaccination and subsequent monitoring of their inhibition of the release of ionized calcium to peripheral blood upon injection of recombinant mOPGL into mice (as described by

5 Burgess et al.).

It should be noted that as an alternative to modified OPGL, antiidiotypic antibodies directed against the idiotype of an anti-OPGL antibody will also serve as useful immunogens within the scope of the present invention. Likewise, the use of

10 mimotypic polypeptides which can be isolated in e.g. a phage display system using anti-OPGL or osteoprotegerin as catching probe are also considered as part of the immunogens of the invention.

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CLAIMS

1. A method for *in vivo* down-regulation of osteoprotegerin
ligand (OPGL) activity in an animal, including a human being,
the method comprising effecting presentation to the animal's
5    immune system of an immunogenically effective amount of
     -    at least one OPGL polypeptide or subsequence thereof
          which has been formulated so that immunization of the
          animal with the OPGL polypeptide or subsequence thereof
          induces production of antibodies against the OPGL
10        polypeptide, and/or
     -    at least one OPGL analogue wherein is introduced at least
          one modification in the OPGL amino acid sequence which
          has as a result that immunization of the animal with the
          analogue induces production of antibodies against the
15        OPGL polypeptide.

2. The method according to claim 1, wherein is presented an
OPGL analogue with at least one modification of the OPGL amino
acid sequence.

3. The method according to claim 2, wherein the modification
20   has as a result that a substantial fraction of OPGL B-cell
epitopes are preserved and that
     -    at least one foreign T helper lymphocyte epitope ($T_H$
          epitope) is introduced, and/or
     -    at least one first moiety is introduced which effects
25        targeting of the modified molecule to an antigen present-
          ing cell (APC) or a B-lymphocyte, and/or
     -    at least one second moiety is introduced which stimulates
          the immune system, and/or
     -    at least one third moiety is introduced which optimizes
30        presentation of the modified OPGL polypeptide to the
          immune system.

4. The method according to claim 3, wherein the modification includes introduction as side groups, by covalent or non-covalent binding to suitable chemical groups in OPGL or a subsequence thereof, of the foreign $T_H$ epitope and/or of the

5 first and/or of the second and/or of the third moiety.

5. The method according to claim 3 or 4, wherein the modification includes amino acid substitution and/or deletion and/or insertion and/or addition.

6. The method according to claim 5, wherein the modification

10 results in the provision of a fusion polypeptide.

7. The method according to claim 5 or 6, wherein introduction of the amino acid substitution and/or deletion and/or insertion and/or addition results in a substantial preservation of the overall tertiary structure of OPGL.

15 8. The method according to any one of claims 2-7, wherein the modification includes duplication of at least one OPGL B-cell epitope and/or introduction of a hapten.

9. The method according to any one of claims 3-8, wherein the foreign T-cell epitope is immunodominant in the animal.

20 10. The method according to any one of claims 3-9, wherein the foreign T-cell epitope is promiscuous.

11. The method according to claim 10, wherein the at least one foreign T-cell epitope is selected from a natural promiscuous T-cell epitope and an artificial MHC-II binding peptide se-

25 quence.

12. The method according to claim 11, wherein the natural T-cell epitope is selected from a Tetanus toxoid epitope such as

P2 or P30, a diphtheria toxoid epitope, an influenza virus
hemagluttinin epitope, and a *P. falciparum* CS epitope.


13. The method according to any one of claims 3-12, wherein
the first moiety is a substantially specific binding partner
5 for a B-lymphocyte specific surface antigen or for an APC
specific surface antigen such as a hapten or a carbohydrate
for which there is a receptor on the B-lymphocyte or the APC.


14. The method according to any one of claims 3-13, wherein
the second moiety is selected from a cytokine, a hormone, and
10 a heat-shock protein.


15. The method according to claim 6, wherein the cytokine is
selected from, or is an effective part of, interferon γ (IFN-
γ), Flt3L, interleukin 1 (IL-1), interleukin 2 (IL-2),
interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12
15 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and
granulocyte-macrophage colony stimulating factor (GM-CSF), and
the heat-shock protein is selected from, or is an effective
part of, HSP70, HSP90, HSC70, GRP94, and calreticulin (CRT).


16. The method according to any one of claims 3-15, wherein
20 the third moiety is of lipid nature, such as a palmitoyl
group, a myristyl group, a farnesyl group, a geranyl-geranyl
group, a GPI-anchor, and an N-acyl diglyceride group.


17. The method according to any one of the preceding claims,
wherein the OPGL polypeptide or subsequence thereof has been
25 modified in any one of positions 170-192, any one of positions
198-218, any one of positions 221-246, any one of positions
256-261, or in any one of positions 285-316, the amino acid
numbering conforming with that of any one of SEQ ID NOs: 4, 6,
and 12, or wherein the OPGL polypeptide has been modified in
30 any one of positions 171-193, any one of positions 199-219,

any one of positions 222-247, any one of positions 257-262, or
in any one of positions 286-317, the amino acid numbering
conforming with that of SEQ ID NO: 2.

18. The method according to claim 17, wherein the modification
5  comprises a substitution of at least one amino acid sequence—
within a position defined in claim 17 with an amino acid
sequence of equal or different length which contains a foreign
$T_H$ epitope.

19. The method according to claim 18, wherein the amino acid
10  sequence containing the foreign $T_H$ epitope substitutes amino
acids 256-261 and/or 288-302 and/or 221-241 found in SEQ ID
NO: 4 or amino acids 257-262 and/or 289-303 and/or 222-243 in
SEQ ID NO: 2 or in a polypeptide where a cysteine correspon-
ding to Cys-221 has been substituted with Ser.

15  20. The method according to any one of the preceding claims,
wherein presentation to the immune system is effected by
having at least two copies of the OPGL polypeptide, the
subsequence thereof or the modified OPGL polypeptide cova-
lently of non-covalently linked to a carrier molecule capable
20  of effecting presentation of multiple copies of antigenic
determinants.

21. The method according to any the preceding claims, wherein
the OPGL polypeptide, the subsequence thereof, or the modified
OPGL polypeptide has been formulated with an adjuvant which
25  facilitates breaking of autotolerance to autoantigens.

22. The method according to any one of the preceding claims,
wherein an effective amount of the OPGL polypeptide or the
OPGL analogue is administered to the animal via a route se-
lected from the parenteral route such as the intradermal, the
30  subdermal, the intracutaneous, the subcutaneous, and the
intramuscular routes; the peritoneal route; the oral route;

the buccal route; the sublingual route; the epidural route; the spinal route; the anal route; and the intracranial route.

23. The method according to claim 22, wherein the effective amount is between 0.5 µg and 2,000 µg of the OPGL polypeptide,
5 the subsequence thereof or the analogue thereof.

24. The method according to claim 22 or 23, wherein the OPGL polypeptide or analogue is contained in a virtual lymph node (VLN) device.

25. The method according to any one of claims 1-21, wherein
10 presentation of modified OPGL to the immune system is effected by introducing nucleic acid(s) encoding the modified OPGL into the animal's cells and thereby obtaining *in vivo* expression by the cells of the nucleic acid(s) introduced.

26. The method according to claim 25, wherein the nucleic
15 acid(s) introduced is/are selected from naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated
20 with Calcium precipitating agents, DNA coupled to an inert carrier molecule, DNA encapsulated in chitin or chitosan, and DNA formulated with an adjuvant.

27. The method according to claim 27, wherein the nucleic acid(s) is/are contained in a VLN device.

25 28. The method according to any one of claims 22-27, which includes at least one administration/introduction per year, such as at least 2, at least 3, at least 4, at least 6, and at least 12 administrations/introductions.

29. A method for treating and/or preventing and/or ameliora-
ting osteoporosis or other diseases and conditions characte-
rized by excess bone resorption, the method comprising down-
regulating OPGL activity according to the method of any one of
5   claims 1-28 to such an extent that the rate of bone resorption
is significantly decreased, such as a decrease of at least 3%,
at least 7%, at least 9%, at least 11%, at least 13%, at least
15%, at least 17%, at least 20%, and at least 30%.


30. An OPGL analogue which is derived from an animal OPGL
10  polypeptide wherein is introduced a modification which has as
a result that immunization of the animal with the analogue
induces production of antibodies against the OPGL polypeptide.


31. An OPGL analogue according to claim 30, wherein the
modification is as defined in any one of claims 1-19.


15  32. An immunogenic composition comprising
-    an immunogenically effective amount of an OPGL polypep-
     tide autologous in an animal, said OPGL polypeptide being
     formulated together with an immunologically acceptable
     adjuvant so as to break the animal's autotolerance to-
20   wards the OPGL polypeptide, the composition further com-
     prising a pharmaceutically and immunologically acceptable
     carrier and/or vehicle, or
-    an immunogenically effective amount of an OPGL analogue
     according to claim 30 or 31, the composition further
25   comprising a pharmaceutically and immunologically accept-
     able carrier and/or vehicle and optionally an adjuvant.


33. A nucleic acid fragment which encodes an OPGL analogue
according to claim 30 or 31.


34. A vector carrying the nucleic acid fragment according to
30  claim 33.

35. The vector according to claim 34 which is capable of
autonomous replication.

36. The vector according to claim 34 or 35 which is selected
from the group consisting of a plasmid, a phage, a cosmid, a
5 mini-chromosome, and a virus.

37. The vector according to any one of claims 34-36, compri-
sing, in the 5'-3' direction and in operable linkage, a pro-
moter for driving expression of the nucleic acid fragment
according to claim 33, optionally a nucleic acid sequence
10 encoding a leader peptide enabling secretion of or integration
into the membrane of the polypeptide fragment, the nucleic
acid fragment according to claim 33, and optionally a termina-
tor.

38. The vector according to any one of claims 34-37 which,
15 when introduced into a host cell, is capable or incapable of
being integrated in the host cell genome.

39. The vector according to claim 37 or 38, wherein a promoter
drives expression in a eukaryotic cell and/or in a prokaryotic
cell.

20 40. A transformed cell carrying the vector of any one of
claims 34-39.

41. The transformed cell according to claim 40 which is capa-
ble of replicating the nucleic acid fragment according to
claim 33.

25 42. The transformed cell according to claim 41, which is a
microorganism selected from a bacterium, a yeast, a protozoan,
or a cell derived from a multicellular organism selected from
a fungus, an insect cell such as an $S_2$ or an SF cell, a plant
cell, and a mammalian cell.

43. The transformed cell according to any one of claims 40-42, which expresses the nucleic acid fragment according to claim 33.

44. The transformed cell according to claim 43, which secretes
5 or carries on its surface, the OPGL analogue according to ⁻ claim 30 or 31.

45. The method according to any one of claims 1-19, wherein presentation to the immune system is effected by administering a non-pathogenic microorganism or virus which is carrying a
10 nucleic acid fragment which encodes and expresses the OPGL polypeptide or analogue.

46. A composition for inducing production of antibodies a-gainst OPGL, the composition comprising
   -      a nucleic acid fragment according to claim 33 or a vector
15         according to any one of claims 34-39, and
   -      a pharmaceutically and immunologically acceptable carrier
          and/or vehicle and/or adjuvant.

47. A stable cell line which carries the vector according to any one of claims 34-39 and which expresses the nucleic acid
20 fragment according to claim 33, and which optionally secretes or carries the OPGL analogue according to claim 30 or 31 on its surface.

48. A method for the preparation of the cell according to any one of claims 40-44, the method comprising transforming a host
25 cell with the nucleic acid fragment according to claim 33 or with the vector according to any one of claims 34-39.

49. A method for the identification of a modified OPGL polypeptide which is capable of inducing antibodies against unmodified OPGL in an animal species where the unmodified OPGL
30 polypeptide is a self-protein, the method comprising

- preparing, by means of peptide synthesis or genetic
  engineering techniques, a set of mutually distinct modi-
  fied OPGL polypeptides wherein amino acids have been
  added to, inserted in, deleted from, or substituted into

5      the amino acid sequence of an OPGL polypeptide of the
       animal species thereby giving rise to amino acid sequen-
       ces in the set which comprise T-cell epitopes which are
       foreign to the animal species, or preparing a set of
       nucleic acid fragments encoding the set of mutually dis-

10     tinct modified OPGL polypeptides,

- testing members of the set of modified OPGL polypeptides
  or nucleic acid fragments for their ability to induce
  production of antibodies by the animal species against
  the unmodified OPGL, and

15 -   identifying and optionally isolating the member(s) of the
       set of modified OPGL polypeptides which significantly
       induces antibody production against unmodified OPGL in
       the species or identifying and optionally isolating the
       polypeptide expression products encoded by members of the

20     set of nucleic acid fragments which significantly induces
       antibody production against unmodified OPGL in the animal
       species.


50. A method for the preparation of an immunogenic composition
comprising at least one modified OPGL polypeptide which is
25 capable of inducing antibodies against unmodified OPGL in an
animal species where the unmodified OPGL polypeptide is a
self-protein, the method comprising

- preparing, by means of peptide synthesis or genetic engi-
  neering techniques, a set of mutually distinct modified

30     OPGL polypeptides wherein amino acids have been added to,
       inserted in, deleted from, or substituted into the amino
       acid sequence of an OPGL polypeptide of the animal spe-
       cies thereby giving rise to amino acid sequences in the

set comprising T-cell epitopes which are foreign to the
animal,
- testing members of the set for their ability to induce
  production of antibodies by the animal species against
5   the unmodified OPGL, and
- admixing the member(s) of the set which significantly
  induces production of antibodies in the animal species
  which are reactive with OPGL with a pharmaceutically and
  immunologically acceptable carrier and/or vehicle, op-
10  tionally in combination with at least one pharmaceuti-
    cally and immunologically acceptable adjuvant.

51. The method according to claim 49 or 50, wherein prepara-
tion of the members of the set comprises preparation of mutu-
ally distinct nucleic acid sequences, each sequence being a
15 nucleic acid sequence according to claim 33, insertion of the
nucleic acid sequences into appropriate expression vectors,
transformation of suitable host cells with the vectors, and
expression of the nucleic acid sequences, optionally followed
by isolation of the expression products.

20 52. The method according to claim 51, wherein the preparation
of the nucleic acid sequences and/or the vectors is achieved
by the aid of a molecular amplification technique such as PCR
or by the aid of nucleic acid synthesis.

53. Use of OPGL or a subsequence thereof for the preparation
25 of an immunogenic composition comprising an adjuvant for down-
regulating OPGL activity in an animal.

54. Use of OPGL or a subsequence thereof for the preparation
of an immunogenic composition comprising an adjuvant for the
treatment, prophylaxis or amelioration of osteoporosis or
30 other conditions characterized by excessive bone resorption.

55. Use of an OPGL analogue for the preparation of an
immunogenic composition optionally comprising an adjuvant for
down-regulating OPGL activity in an animal.

56. Use of an OPGL analogue for the preparation of an
5   immunogenic composition optionally comprising an adjuvant for
the treatment, prophylaxis or amelioration of osteoporosis or
other conditions characterized by excessive bone resorption.

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      HALKIER, Torben
      HAANING, Jesper

<120> Method for Down-Regulating Osteoprotegerin Ligand
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<130> 22021 PC 1

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Glu Glu Met Gly Gly Gly Pro Gly Ala Pro His Glu Gly Pro Leu His
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gcc ccg ccg ccg cct gcg ccg cac cag ccc ccc gcc gcc tcc cgc tcc 325
Ala Pro Pro Pro Pro Ala Pro His Gln Pro Pro Ala Ala Ser Arg Ser
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Met Phe Val Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser
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gtc gcc ctg ttc ttc tat ttc aga gcg cag atg gat cct aat aga ata       421
Val Ala Leu Phe Phe Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile
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tca gaa gat ggc act cac tgc att tat aga att ttg aga ctc cat gaa       469
Ser Glu Asp Gly Thr His Cys Ile Tyr Arg Ile Leu Arg Leu His Glu
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aat gca gat ttt caa gac aca act ctg gag agt caa gat aca aaa tta       517
Asn Ala Asp Phe Gln Asp Thr Thr Leu Glu Ser Gln Asp Thr Lys Leu
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ata cct gat tca tgt agg aga att aaa cag gcc ttt caa gga gct gtg       565
Ile Pro Asp Ser Cys Arg Arg Ile Lys Gln Ala Phe Gln Gly Ala Val
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caa aag gaa tta caa cat atc gtt gga tca cag cac atc aga gca gag       613
Gln Lys Glu Leu Gln His Ile Val Gly Ser Gln His Ile Arg Ala Glu
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aaa gcg atg gtg gat ggc tca tgg tta gat ctg gcc aag agg agc aag       661
Lys Ala Met Val Asp Gly Ser Trp Leu Asp Leu Ala Lys Arg Ser Lys
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ctt gaa gct cag cct ttt gct cat ctc act att aat gcc acc gac atc       709
Leu Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Thr Asp Ile
160             165             170             175

cca tct ggt tcc cat aaa gtg agt ctg tcc tct tgg tac cat gat cgg       757
Pro Ser Gly Ser His Lys Val Ser Leu Ser Ser Trp Tyr His Asp Arg
            180             185             190

ggt tgg gcc aag atc tcc aac atg act ttt agc aat gga aaa cta ata       805
Gly Trp Ala Lys Ile Ser Asn Met Thr Phe Ser Asn Gly Lys Leu Ile
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cat cat gaa act tca gga gac cta gct aca gag tat ctt caa cta atg       901
His His Glu Thr Ser Gly Asp Leu Ala Thr Glu Tyr Leu Gln Leu Met
        225             230             235

gtg tac gtc act aaa acc agc atc aaa atc cca agt tct cat acc ctg       949
Val Tyr Val Thr Lys Thr Ser Ile Lys Ile Pro Ser Ser His Thr Leu
240             245             250             255

atg aaa gga gga agc acc aag tat tgg tca ggg aat tct gaa ttc cat       997
Met Lys Gly Gly Ser Thr Lys Tyr Trp Ser Gly Asn Ser Glu Phe His
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gaa atc agc atc gag gtc tcc aac ccc tcc tta ctg gat ccg gat cag   1093
Glu Ile Ser Ile Glu Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln
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gat gca aca tac ttt ggg gct ttt aaa gtt cga gat ata gat tga       1138
Asp Ala Thr Tyr Phe Gly Ala Phe Lys Val Arg Asp Ile Asp
        305                 310                 315
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        35                  40                  45

Phe Val Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser Val
    50                  55                  60

Ala Leu Phe Phe Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser
65                  70                  75                  80

Glu Asp Gly Thr His Cys Ile Tyr Arg Ile Leu Arg Leu His Glu Asn
                85                  90                  95

Ala Asp Phe Gln Asp Thr Thr Leu Glu Ser Gln Asp Thr Lys Leu Ile
            100                 105                 110

Pro Asp Ser Cys Arg Arg Ile Lys Gln Ala Phe Gln Gly Ala Val Gln
        115                 120                 125

Lys Glu Leu Gln His Ile Val Gly Ser Gln His Ile Arg Ala Glu Lys
    130                 135                 140

Ala Met Val Asp Gly Ser Trp Leu Asp Leu Ala Lys Arg Ser Lys Leu
145                 150                 155                 160

Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Thr Asp Ile Pro
                165                 170                 175

Ser Gly Ser His Lys Val Ser Leu Ser Ser Trp Tyr His Asp Arg Gly
            180                 185                 190

Trp Ala Lys Ile Ser Asn Met Thr Phe Ser Asn Gly Lys Leu Ile Val
        195                 200                 205

Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
    210                 215                 220

His Glu Thr Ser Gly Asp Leu Ala Thr Glu Tyr Leu Gln Leu Met Val
225                 230                 235                 240

Tyr Val Thr Lys Thr Ser Ile Lys Ile Pro Ser Ser His Thr Leu Met
                245                 250                 255

Lys Gly Gly Ser Thr Lys Tyr Trp Ser Gly Asn Ser Glu Phe His Phe
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                 260                265                270

    Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ser Gly Glu Glu
            275                280                285


    Ile Ser Ile Glu Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
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    Ala Thr Tyr Phe Gly Ala Phe Lys Val Arg Asp Ile Asp
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Glu Met Gly Ser Gly Pro Gly Val Pro His Glu Gly Pro Leu His Pro
            20                25                30

gcg cct tct gca ccg gct ccg gcg ccg cca ccc gcc gcc tcc cgc tcc     144
Ala Pro Ser Ala Pro Ala Pro Ala Pro Pro Pro Ala Ala Ser Arg Ser
            35                40                45

atg ttc ctg gcc ctc ctg ggg ctg gga ctg ggc cag gtg gtc tgc agc     192
Met Phe Leu Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser
        50                55                60

atc gct ctg ttc ctg tac ttt cga gcg cag atg gat cct aac aga ata     240
Ile Ala Leu Phe Leu Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile
    65                70                75                80
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6

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tca gaa gac agc act cac tgc ttt tat aga atc ctg aga ctc cat gaa      288
Ser Glu Asp Ser Thr His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu
                85                  90                  95

aac gca ggt ttg cag gac tcg act ctg gag agt gaa gac aca cta cct      336
Asn Ala Gly Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro
            100                 105                 110

gac tcc tgc agg agg atg aaa caa gcc ttt cag ggg gcc gtg cag aag      384
Asp Ser Cys Arg Arg Met Lys Gln Ala Phe Gln Gly Ala Val Gln Lys
        115                 120                 125

gaa ctg caa cac att gtg ggg cca cag cgc ttc tca gga gct cca gct      432
Glu Leu Gln His Ile Val Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala
    130                 135                 140

atg atg gaa ggc tca tgg ttg gat gtg gcc cag cga ggc aag cct gag      480
Met Met Glu Gly Ser Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu
145                 150                 155                 160

gcc cag cca ttt gca cac ctc acc atc aat gct gcc agc atc cca tcg      528
Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser
                165                 170                 175

ggt tcc cat aaa gtc act ctg tcc tct tgg tac cac gat cga ggc tgg      576
Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp
            180                 185                 190

gcc aag atc tct aac atg acg tta agc aac gga aaa cta agg gtt aac      624
Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn
        195                 200                 205

caa gat ggc ttc tat tac ctg tac gcc aac att tgc ttt cgg cat cat      672
Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His
    210                 215                 220

gaa aca tcg gga agc gta cct aca gac tat ctt cag ctg atg gtg tat      720
Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr
225                 230                 235                 240

gtc gtt aaa acc agc atc aaa atc cca agt tct cat aac ctg atg aaa      768
Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys
                245                 250                 255

gga ggg agc acg aaa aac tgg tcg ggc aat tct gaa ttc cac ttt tat      816
Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
            260                 265                 270

tcc ata aat gtt ggg gga ttt ttc aag ctc cga gct ggt gaa gaa att      864
Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
        275                 280                 285
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agc att cag gtg tcc aac cct tcc ctg ctg gat ccg gat caa gat gcg    912
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    290             295             300

acg tac ttt ggg gct ttc aaa gtt cag gac ata gac tga             951
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305             310             315
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        35              40              45

Met Phe Leu Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser
    50              55              60

Ile Ala Leu Phe Leu Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile
65              ·70              75              80

Ser Glu Asp Ser Thr His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu
            85              90              95

Asn Ala Gly Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro
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Asp Ser Cys Arg Arg Met Lys Gln Ala Phe Gln Gly Ala Val Gln Lys
        115             120             125

Glu Leu Gln His Ile Val Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala
    130             135             140

Met Met Glu Gly Ser Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu
145             150             155             160

Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser
            165             170             175

Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp
            180             185             190

Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn
        195             200             205
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8

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Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His
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Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr
225             230             235             240
Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys
            245             250             255
Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
        260             265             270
Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
        275             280             285
Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala
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Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
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gcc agc cga gac tac ggc aag tac ctg cgc agc tcg gag gag atg ggc  226
Ala Ser Arg Asp Tyr Gly Lys Tyr Leu Arg Ser Ser Glu Glu Met Gly
      5              10              15

agc ggc ccc ggc gtc cca cac gag ggt ccg ctg cac ccc gcg cct tct  274
Ser Gly Pro Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser
 20              25              30              35

gca ccg gct ccg gcg ccg cca ccc gcc gcc tcc cgc tcc atg ttc ctg  322
Ala Pro Ala Pro Ala Pro Pro Pro Ala Ala Ser Arg Ser Met Phe Leu
            40              45              50
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gcc ctc ctg ggg ctg gga ctg ggc cag gtg gtc tgc agc atc gct ctg    370
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ttc ctg tac ttt cga gcg cag atg gat cct aac aga ata tca gaa gac    418
Phe Leu Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser Glu Asp
            70                  75                  80


agc act cac tgc ttt tat aga atc ctg aga ctc cat gaa aac gca ggt    466
Ser Thr His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Gly
         85                  90                  95


ttg cag gac tcg act ctg gag agt gaa gac aca cta cct gac tcc tgc    514
Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys
100                 105                 110                 115


agg agg atg aaa caa gcc ttt cag ggg gcc gtg cag aag gaa ctg caa    562
Arg Arg Met Lys Gln Ala Phe Gln Gly Ala Val Gln Lys Glu Leu Gln
                120                 125                 130


cac att gtg ggg cca cag cgc ttc tca gga gct cca gct atg atg gaa    610
His Ile Val Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala Met Met Glu
                135                 140                 145


ggc tca tgg ttg gat gtg gcc cag cga ggc aag cct gag gcc cag cca    658
Gly Ser Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu Ala Gln Pro
            150                 155                 160


ttt gca cac ctc acc atc aat gct gcc agc atc cca tcg ggt tcc cat    706
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aaa gtc act ctg tcc tct tgg tac cac gat cga ggc tgg gcc aag atc    754
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tct aac atg acg tta agc aac gga aaa cta agg gtt aac caa gat ggc    802
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ttc tat tac ctg tac gcc aac att tgc ttt cgg cat cat gaa aca tcg    850
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gga agc gta cct aca gac tat ctt cag ctg atg gtg tat gtc gtt aaa    898
Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr Val Val Lys
                230                 235                 240


acc agc atc aaa atc cca agt tct cat aac ctg atg aaa gga ggg agc    946
Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly Gly Ser
            245                 250                 255
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acg aaa aac tgg tcg ggc aat tct gaa ttc cac ttt tat tcc ata aat   994
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gtt ggg gga ttt ttc aag ctc cga gct ggt gaa gaa att agc att cag   1042
Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln
                280             285             290

gtg tcc aac cct tcc ctg ctg gat ccg gat caa gat gcg acg tac ttt   1090
Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe
            295             300             305

ggg gct ttc aaa gtt cag gac ata gac tga gactcatttc gtggaacatt     1140
Gly Ala Phe Lys Val Gln Asp Ile Asp
            310             315
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             20                  25                  30

Ala Pro Ser Ala Pro Ala Pro Ala Pro Pro Pro Ala Ala Ser Arg Ser
         35                  40                  45

Met Phe Leu Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser
     50                  55                  60

Ile Ala Leu Phe Leu Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile
 65                  70                  75                  80

Ser Glu Asp Ser Thr His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu
                 85                  90                  95

Asn Ala Gly Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro
             100                 105                 110

Asp Ser Cys Arg Arg Met Lys Gln Ala Phe Gln Gly Ala Val Gln Lys
         115                 120                 125

Glu Leu Gln His Ile Val Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala
     130                 135                 140

Met Met Glu Gly Ser Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu
145                 150                 155                 160

Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser
                 165                 170                 175

Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp
             180                 185                 190

Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn
             195                 200                 205

Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His
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Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr
225                 230                 235                 240

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Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys
            245                 250                 255

Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
            260                 265                 270

Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
        275                 280                 285

Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala
    290                 295                 300

Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
305                 310                 315
```


```
<210> 7
<211> 564
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)..(564)

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      product with optimum codons for E. coli and P.
      pastoris expression

<220>
<221> misc_binding
<222> (43)..(84)
<223> His tag

<220>
<221> misc_feature
<222> (1)..(36)
<223> C-terminal part of Saccharomyces cerevisiae
      alpha-mating factor

<220>
<221> misc_feature
<222> (85)..(561)
<223> Encoding wild type murine OPGL, residues 158-316

<400> 7
gag ctc gga tcc ctc gag aaa aga gag gct gaa gct cat gtc atg aaa    48
Glu Leu Gly Ser Leu Glu Lys Arg Glu Ala Glu Ala His Val Met Lys
    1               5                   10                  15
```

```
cac caa cac caa cat caa cat caa cat caa cat caa aaa cct gaa gct    96
His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro Glu Ala
            20              25              30

cag cca ttc gct cat ctg acc atc aac gct gca tcg atc cct tct ggt   144
Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser Gly
        35              40              45

tct cat aaa gtt acc ctg tct tct tgg tat cac gac cgc ggt tgg gct   192
Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala
    50              55              60

aaa atc tct aac atg acc ctg tct aac ggt aaa ctg aga gtt aac cag   240
Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn Gln
65              70              75              80

gac ggt ttc tac tac ctg tac gct aac atc tgt ttc aga cat cac gaa   288
Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His Glu
            85              90              95

acc tct ggt tct gtt cca acc gac tac ctg cag ctg atg gtt tac gtt   336
Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr Val
        100             105             110

gtt aaa acc tct atc aaa atc cca tct tca cat aac ctg atg aaa ggt   384
Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly
        115             120             125

ggt tct acc aaa aac tgg tct ggt aac tct gaa ttc cat ttc tac tct   432
Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser
    130             135             140

atc aac gtt ggt ggt ttc ttc aaa ctg aga gct ggt gaa gaa atc tct   480
Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser
145             150             155             160

atc cag gtt tct aac cct tct ctg ctg gac cca gac cag gac gct acc   528
Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr
            165             170             175

tac ttc ggg gcc ttc aaa gtt cag gac atc gac tag               564
Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
        180             185
```

```
<210> 8
<211> 187
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic PCR
      product with optimum codons for E. coli and P.
      pastoris expression
```

<400> 8
Glu Leu Gly Ser Leu Glu Lys Arg Glu Ala Glu Ala His Val Met Lys
 1               5                  10                  15

His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro Glu Ala
                20                  25                  30

Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser Gly
         35                  40                  45

Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala
         50                  55                  60

Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn Gln
 65                  70                  75                  80

Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His Glu
                85                  90                  95

Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr Val
                100                 105                 110

Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly
         115                 120                 125

Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser
         130                 135                 140

Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser
145                 150                 155                 160

Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr
                165                 170                 175

Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
                180                 185


<210> 9
<211> 519
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: DNA encoding
      murine OPGL, residues 158-316, fused to His tag

<220>
<221> CDS
<222> (1)..(519)

<220>

<221> misc_binding
<222> (1)..(42)
<223> His tag

<220>
<221> misc_feature
<222> (43)..(519)
<223> Murine OPGL, residues 158-316

<400> 9
```
atg aaa cac caa cac caa cat caa cat caa cat caa cat caa aaa cct    48
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5                  10                  15

gaa gct cag cca ttc gct cat ctg acc atc aac gct gca tcg atc cct    96
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20                  25                  30

tct ggt tct cat aaa gtt acc ctg tct tct tgg tat cac gac cgc ggt   144
Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
        35                  40                  45

tgg gct aaa atc tct aac atg acc ctg tct aac ggt aaa ctg aga gtt   192
Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50                  55                  60

aac cag gac ggt ttc tac tac ctg tac gct aac atc tgt ttc aga cat   240
Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
 65                  70                  75                  80

cac gaa acc tct ggt tct gtt cca acc gac tac ctg cag ctg atg gtt   288
His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                    85                  90                  95

tac gtt gtt aaa acc tct atc aaa atc cca tct tca cat aac ctg atg   336
Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
                100                 105                 110

aaa ggt ggt tct acc aaa aac tgg tct ggt aac tct gaa ttc cat ttc   384
Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
            115                 120                 125

tac tct atc aac gtt ggt ggt ttc ttc aaa ctg aga gct ggt gaa gaa   432
Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
        130                 135                 140

atc tct atc cag gtt tct aac cct tct ctg ctg gac cca gac cag gac   480
Ile Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
145                 150                 155                 160

gct acc tac ttc ggg gcc ttc aaa gtt cag gac atc gac               519
Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
                165                 170
```

```
<210> 10
<211> 173
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: DNA encoding
      murine OPGL, residues 158-316, fused to His tag

<400> 10
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5                  10                  15

Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
             20                  25                  30

Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
         35                  40                  45

Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
     50                  55                  60

Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
 65                  70                  75                  80

His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                 85                  90                  95

Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
                 100                 105                 110

Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
             115                 120                 125

Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
     130                 135                 140

Ile Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
145                 150                 155                 160

Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
                 165                 170


<210> 11
<211> 519
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Fusion of
      murine OPGL, residues 158-316 with C to S
      mutation,  and His tag
```

17

```
<220>
<221> CDS
<222> (1)..(519)

<220>
<221> misc_binding
<222> (1)..(42)
<223> His tag

<220>
<221> misc_feature
<222> (43)..(228)
<223> Murine OPGL, residues 158-219

<220>
<221> misc_feature
<222> (232)..(519)
<223> Murine OPGL, residues 221-316

<220>
<221> mutation
<222> (229)..(231)
<223> tgt (Cys) to tcc (Ser)

<220>

<400> 11
atg aaa cac caa cac caa cat caa cat caa cat caa cat caa aaa cct    48
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5                  10                  15

gaa gct cag cca ttc gct cat ctg acc atc aac gct gca tcg atc cct    96
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
                20                  25                  30

tct ggt tct cat aaa gtt acc ctg tct tct tgg tat cac gac cgc ggt   144
Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
            35                  40                  45

tgg gct aaa atc tct aac atg acc ctg tct aac ggt aaa ctg aga gtt   192
Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
        50                  55                  60

aac cag gac ggt ttc tac tac ctg tac gct aac atc tcc ttc aga cat   240
Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Ser Phe Arg His
65                  70                  75                  80

cac gaa acc tct ggt tct gtt cca acc gac tac ctg cag ctg atg gtt   288
His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                85                  90                  95
```

18

```
tac gtt gtt aaa acc tct atc aaa atc cca tct tca cat aac ctg atg    336
Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
            100                 105                 110


aaa ggt ggt tct acc aaa aac tgg tct ggt aac tct gaa ttc cat ttc    384
Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
            115                 120                 125


tac tct atc aac gtt ggt ggt ttc ttc aaa ctg aga gct ggt gaa gaa    432
Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
            130                 135                 140


atc tct atc cag gtt tct aac cct tct ctg ctg gac cca gac cag gac    480
Ile Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
145                 150                 155                 160


gct acc tac ttc ggg gcc ttc aaa gtt cag gac atc gac               519
Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
            165                 170
```


<210> 12
<211> 173
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:  Fusion of
      murine OPGL, residues 158-316 with C to S
      mutation,  and His tag

<400> 12
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
  1               5                  10                  15


Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20                  25                  30


Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
            35                  40                  45


Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
      50                  55                  60


Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Ser Phe Arg His
65                  70                  75                  80


His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                  85                  90                  95


Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
            100                 105                 110


Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
            115                 120                 125

Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
    130                 135                 140

Ile Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
145                 150                 155                 160

Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
                165                 170                                    —


<210> 13
<211> 564
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion of
      murine OPGL, residues 158-316 modified by
      introduction of tetanus toxoid P30 epitope, and
      His tag

<220>
<221> CDS
<222> (1)..(564)

<220>
<221> misc_binding
<222> (1)..(42)
<223> His tag

<220>
<221> misc_feature
<222> (43)..(336)
<223> Murine OPGL, residues 158-255

<220>
<221> misc_feature
<222> (337)..(399)
<223> Tetanus toxoid P30 epitope

<220>
<221> misc_feature
<222> (400)..(564)
<223> Murine OPGL, residues 262-316

<400> 13
atg aaa cac caa cac caa cat caa cat caa cat caa cat caa aaa cct     48
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
  1               5                   10                  15

```
gaa gct cag cca ttc gct cat ctg acc atc aac gct gca tcg atc cct      96
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20              25              30

tct ggt tct cat aaa gtt acc ctg tct tct tgg tat cac gac cgc ggt     144
Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
        35              40              45

tgg gct aaa atc tct aac atg acc ctg tct aac ggt aaa ctg aga gtt     192
Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50              55              60

aac cag gac ggt ttc tac tac ctg tac gct aac atc tgt ttc aga cat     240
Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
65              70              75              80

cac gaa acc tct ggt tct gtt cca acc gac tac ctg cag ctg atg gtt     288
His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                85              90              95

tac gtt gtt aaa acc tct atc aaa atc cca tct tca cat aac ctg atg     336
Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
            100             105             110

ttc aac aac ttc acc gtt tct ttc tgg ctg agg gta ccg aaa gtt tct     384
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
        115             120             125

gct tct cac ctg gaa aac tgg tct ggt aac tct gaa ttc cat ttc tac     432
Ala Ser His Leu Glu Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
    130             135             140

tct atc aac gtt ggt ggt ttc ttc aaa ctg aga gct ggt gaa gaa atc     480
Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
145             150             155             160

tct atc cag gtt tct aac cct tct ctg ctg gac cca gac cag gac gct     528
Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala
                165             170             175

acc tac ttc ggg gcc ttc aaa gtt cag gac atc gac                     564
Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
            180             185
```

```
<210> 14
<211> 188
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Fusion of
      murine OPGL, residues 158-316 modified by
      introduction of tetanus toxoid P30 epitope, and
      His tag
```

```
<400> 14
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5                  10                 15

Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20                  25                  30

Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
        35                  40                  45

Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50                  55                  60

Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
65                  70                  75                  80

His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                85                  90                  95

Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
            100                 105                 110

Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
            115                 120                 125

Ala Ser His Leu Glu Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
    130                 135                 140

Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
145                 150                 155                 160

Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala
            165                 170                 175

Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
            180                 185


<210> 15
<211> 546
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion
      between murine OPGL, residues 158-316 with tetanus
      toxoid P2 epitope introduced, and His tag

<220>
<221> CDS
<222> (1)..(546)
```

```
<220>
<221> misc_binding
<222> (1)..(42)
<223> His tag

<220>
<221> misc_feature
<222> (43)..(336)
<223> Murine OPGL, residues 158-255

<220>
<221> misc_feature
<222> (382)..(546)
<223> Murine OPGL, residues 262-316

<220>
<221> misc_feature
<222> (337)..(381)
<223> Tetanus toxoid P2 epitope

<400> 15
atg aaa cac caa cac caa cat caa cat caa cat caa cat caa aaa cct    48
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5                  10                  15

gaa gct cag cca ttc gct cat ctg acc atc aac gct gca tcg atc cct    96
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20                  25                  30

tct ggt tct cat aaa gtt acc ctg tct tct tgg tat cac gac cgc ggt   144
Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
        35                  40                  45

tgg gct aaa atc tct aac atg acc ctg tct aac ggt aaa ctg aga gtt   192
Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50                  55                  60

aac cag gac ggt ttc tac tac ctg tac gct aac atc tgt ttc aga cat   240
Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
 65                  70                  75                  80

cac gaa acc tct ggt tct gtt cca acc gac tac ctg cag ctg atg gtt   288
His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                85                  90                  95

tac gtt gtt aaa acc cct atc aaa atc caa tct tca cat aac ctg atg   336
Tyr Val Val Lys Thr Pro Ile Lys Ile Gln Ser Ser His Asn Leu Met
            100                 105                 110

cag tac atc aaa gct aat tcg aaa ttc atc ggt atc acc gaa ctg aac   384
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Asn
            115                 120                 125
```

```
tgg tct ggt aac tct gaa ttc cat ttc tac tct atc aac gtt ggt ggt     432
Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly Gly
    130             135             140

ttc ttc aaa ctg aga gct ggt gaa gaa atc tct atc cag gtt tct aac     480
Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln Val Ser Asn
145             150             155             160

cct tct ctg ctg gac cca gac cag gac gct acc tac ttc ggg gcc ttc     528
Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala Phe
            165             170             175

aaa gtt cag gac atc gac                                             546
Lys Val Gln Asp Ile Asp
        180
```

```
<210> 16
<211> 182
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:  Fusion
      between murine OPGL, residues 158-316 with tetanus
      toxoid P2 epitope introduced, and His tag

<400> 16
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5               10              15

Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20              25              30

Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
        35              40              45

Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50              55              60

Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
 65              70              75              80

His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                85              90              95

Tyr Val Val Lys Thr Pro Ile Lys Ile Gln Ser Ser His Asn Leu Met
            100             105             110

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Asn
        115             120             125

Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly Gly
    130             135             140
```

24

Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln Val Ser Asn
145                 150             155             160

Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala Phe
                165             170             175

Lys Val Gln Asp Ile Asp
            180


<210> 17
<211> 519
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion between
      murine OPGL, residues 158-316 with tetanus toxoid
      P2 epitope introduced, and His tag

<220>
<221> CDS
<222> (1)..(519)

<220>
<221> misc_binding
<222> (1)..(42)
<223> His tag

<220>
<221> misc_feature
<222> (43)..(432)
<223> Murine OPGL, residues 158-287

<220>
<221> misc_feature
<222> (478)..(519)
<223> Murine OPGL, residues 303-316

<220>
<221> misc_feature
<222> (433)..(477)
<223> Tetanus toxoid P2 epitope

<400> 17
atg aaa cac caa cac caa cat caa cat caa cat caa cat caa aaa cct      48
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
  1               5              10              15

gaa gct cag cca ttc gct cat ctg acc atc aac gct gca tcg atc cct      96
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
          20              25              30

25

```
tct ggt tct cat aaa gtt acc ctg tct tct tgg tat cac gac cgc ggt    144
Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
        35              40              45

tgg gct aaa atc tct aac atg acc ctg tct aac ggt aaa ctg aga gtt    192
Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50              55              60

aac cag gac ggt ttc tac tac ctg tac gct aac atc tgt ttc aga cat    240
Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
    65              70              75              80

cac gaa acc tct ggt tct gtt cca acc gac tac ctg cag ctg atg gtt    288
His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                85              90              95

tac gtt gtt aaa acc tct atc aaa atc cca tct tca cat aac ctg atg    336
Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
            100             105             110

aaa ggt ggt tct acc aaa aac tgg tct ggt aac tct gaa ttc cat ttc    384
Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
            115             120             125

tac tct atc aac gtt ggt ggt ttc ttc aaa ctg aga gct ggt gaa gaa    432
Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
    130             135             140

cag tac atc aaa gct aat tcg aaa ttc atc ggt atc acc gaa ctg gac    480
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Asp
145             150             155             160

gct acc tac ttc ggg gcc ttc aaa gtt cag gac atc gac                519
Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
            165             170
```

```
<210> 18
<211> 173
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Fusion between
      murine OPGL, residues 158-316 with tetanus toxoid
      P2 epitope introduced, and His tag

<400> 18
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
1               5               10              15

Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20              25              30

Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
```

26

```
                35                    40                    45

Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50                  55                  60

Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
65                  70                  75                  80

His Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val
                85                  90                  95

Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
                100                 105                 110

Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
            115                 120                 125

Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
    130                 135                 140

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Asp
145                 150                 155                 160

Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
                165                 170
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<210> 19
<211> 519
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion between
     murine OPGL, residues 158-316 with tetanus toxoid
     P30 epitope introduced, and His tag

<220>
<221> CDS
<222> (1)..(519)

<220>
<221> misc_binding
<222> (1)..(42)
<223> His tag

<220>
<221> misc_feature
<222> (43)..(231)
<223> Murine OPGL, residues 158-220

<220>

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<221> misc_feature
<222> (295)..(519)
<223> Murine OPGL, residues 242-316

<220>
<221> misc_feature
<222> (232)..(294)
<223> Tetanus toxoid P30 epitope

<400> 19
atg aaa cac caa cac caa cat caa cat caa cat caa cat caa aaa cct    48
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5                  10                  15

gaa gct cag cca ttc gct cat ctg acc atc aac gct gca tcg atc cct    96
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
                20                  25                  30

tct ggt tct cat aaa gtt acc ctg tct tct tgg tat cac gac cgc ggt    144
Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
            35                  40                  45

tgg gct aaa atc tct aac atg acc ctg tct aac ggt aaa ctg aga gtt    192
Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
        50                  55                  60

aac cag gac ggt ttc tac tac ctg tac gct aac atc tgt ttc aac aac    240
Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Asn Asn
 65                  70                  75                  80

ttc acc gtt tct ttc tgg ctg agg gta ccg aaa gtt tct gct tct cac    288
Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His
                85                  90                  95

ctg gaa gtt aaa acc tct atc aaa atc cca tct tca cat aac ctg atg    336
Leu Glu Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
                100                 105                 110

aaa ggt ggt tct acc aaa aac tgg tct ggt aac tct gaa ttc cat ttc    384
Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
            115                 120                 125

tac tct atc aac gtt ggt ggt ttc ttc aaa ctg aga gct ggt gaa gaa    432
Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
        130                 135                 140

atc tct atc cag gtt tct aac cct tct ctg ctg gac cca gac cag gac    480
Ile Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
145                 150                 155                 160

gct acc tac ttc ggg gcc ttc aaa gtt cag gac atc gac               519
Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
                165                 170
```

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<210> 20
<211> 173
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Fusion between
      murine OPGL, residues 158-316 with tetanus toxoid
      P30 epitope introduced, and His tag
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<400> 20
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Lys Pro
 1               5                   10                  15


Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro
            20                  25                  30


Ser Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly
        35                  40                  45


Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val
    50                  55                  60


Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Asn Asn
65                  70                  75                  80


Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His
                85                  90                  95


Leu Glu Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met
            100                 105                 110


Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe
        115                 120                 125


Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu
    130                 135                 140


Ile Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
145                 150                 155                 160


Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
                165                 170
```

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<210> 21
<211> 68
<212> DNA
<213> Artificial Sequence
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<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer
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<400> 21
agctgcaggt agtcggttgg aacagaacca gaggtttcgt gatgtctgaa acagatgtta 60
gcgtacag                                                           68


<210> 22
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 22
ctcatctgac catcaacgct gcat                                          24


<210> 23
<211> 64
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 23
tttcggtacc ctcagccaga aagaaacggt gaagttgttg aaacagatgt tagcgtacag 60
gtag                                                               64


<210> 24
<211> 61
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 24
tgagggtacc gaaagtttct gcttctcacc tggaagttaa aacccctatc aaaatccaat 60
c                                                                  61


<210> 25
<211> 63
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR

primer

<400> 25
tttcggtacc ctcagccaga aagaaacggt gaagttgttg aacatcaggt tatgtgaaga 60
ttg                                                                63


<210> 26
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 26
tgagggtacc gaaagtttct gcttctcacc tggaaaactg gtctggtaac tctgaattcc 60
at                                                                62


<210> 27
<211> 79
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 27
tacctgcagc tgatggttta cgttgttaaa acccctatca aaatccaatc ttcacataac 60
ctgatgcagt acatcaaag                                               79


<210> 28
<211> 83
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 28
tggaattcag agttaccaga ccagttcagt tcggtgatac cgatgaattt cgaattagct 60
ttgatgtact gcatcaggtt atg                                          83


<210> 29
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 29
gaatttcgaa ttagctttga tgtactgttc ttcaccagct ctcagtttg                    49


<210> 30
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 30
gctaattcga aattcatcgg tatcaccgaa ctggacgcta cctacttcgg ggc               53


<210> 31
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 31
cttactagtc gatgtcctga actttg                                             26


<210> 32
<211> 74
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic PCR
      primer

<400> 32
agtggaattc agagttacca gaccagtttt tggtagaacc acctttcatc aggttatgtg 60
aagatgggat tttg                                                          74


<210> 33
<211> 65
<212> DNA
<213> Clostridium tetani

<400> 33
actacctgca gctgatggtt tacgttgtta aaacctctat caaaatccca tcttcacata 60
acctg                                                                              65


<210> 34
<211> 15
<212> PRT
<213> Clostridium tetani

<400> 34
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 1               5                   10                  15


<210> 35
<211> 21
<212> PRT
<213> Clostridium tetani

<400> 35
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
 1               5                   10                  15

Ala Ser His Leu Glu
        20

# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12N15/62    C12N15/86    C12N15/12    C12N5/10    C12N1/21
       C12N1/19    C07K14/705    A61K39/00    A61K31/713    G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12N   C07K   A61K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched       —

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | FULLER K ET AL: "TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts" J EXP MED, vol. 188, no. 5, 7 September 1998 (1998-09-07), pages 997-1001, XP000876582 cited in the application | |
| A | WO 98 28426 A (IMMUNEX CORP) 2 July 1998 (1998-07-02) claim 34; examples 10,13 | |
| A | WO 98 25958 A (SCHERING CORP) 18 June 1998 (1998-06-18) | |

-/--

[X] Further documents are listed in the continuation of box C.      [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the International search | Date of mailing of the International search report |
|---|---|
| 9 February 2000 | 28/02/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016 | Lonnoy, O |

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1

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category * | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
| A | WO 97 23614 A (AMGEN INC ;LACEY DAVID L (US); BOYLE WILLIAM J (US); CALZONE FRANK) 3 July 1997 (1997-07-03) cited in the application | |
| A | WO 97 20063 A (UNIV COLUMBIA ;BIOGEN INC (US)) 5 June 1997 (1997-06-05) abstract | — |
| A | WO 95 27058 A (UNIV UTAH ;RECHSTEINER MARTIN C (US); REALINI CLAUDIO A (US)) 12 October 1995 (1995-10-12) | |
| P,X | WO 98 46751 A (AMGEN INC ;BOYLE WILLIAM J (US)) 22 October 1998 (1998-10-22) claims 35,36 | 1,53-56 |

1

# INTERNATIONAL SEARCH REPORT

## Box I    Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 1-29 and 45
   are directed to a method of treatment of the human/animal
   body, the search has been carried out and based on the alleged
   effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II    Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest                    [ ] The additional search fees were accompanied by the applicant's protest.

                                     [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
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